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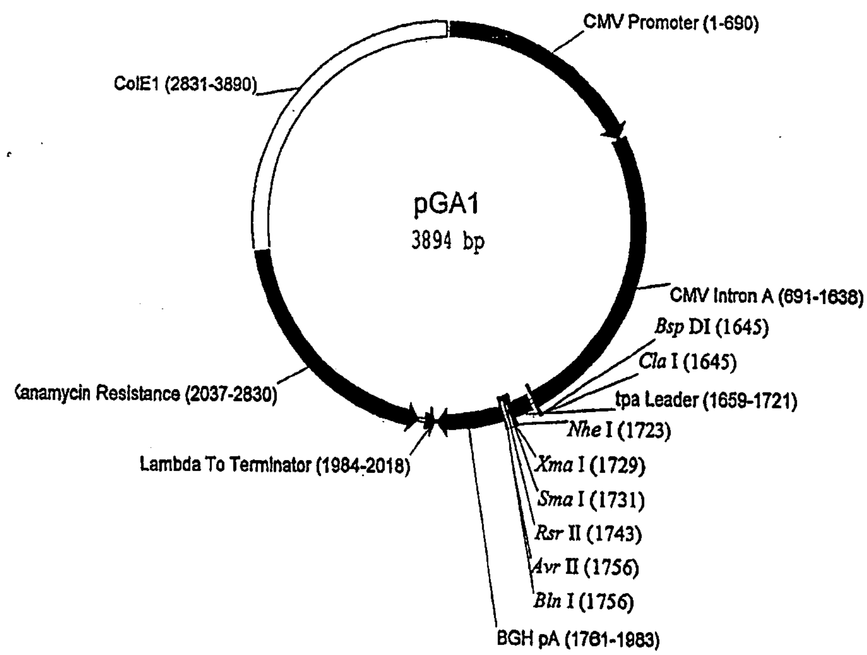
(54) Title: DNA EXPRESSION VECTORS AND METHODS OF USE



(57) Abstract: The present invention provides novel pGA constructs which are useful as vectors for the delivery of DNA vaccine inserts. The vaccine inserts can include the DNA transcripts of various virus, bacteria, parasite and/or fungi. Also described are methods of raising multi-epitope CD8 T-cell responses by administering therapeutically effective amounts of the novel pGA constructs comprising pathogen vaccine inserts followed by booster immunizations with a live vectored vaccine comprising the same vaccine inserts.

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DNA EXPRESSION VECTORS AND METHODS OF USE

The present application claims the benefit of priority from U.S. provisional applications Serial No. 60/186,364, filed March 2, 2000, and Serial No. 60/251,083, filed December 1, 2000.

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FIELD OF THE INVENTION

The present invention is directed generally to the fields of molecular genetics and immunology. More particularly, the present invention describes novel DNA expression vectors, novel vectors comprising DNA encoding an

immunogenic protein, and novel methods of immunizing animals including humans by administering the novel vectors comprising DNA encoding an immunogenic protein.

BACKGROUND OF THE INVENTION

Vaccines have had profound and long lasting effects on world health. Small pox has been eradicated, polio is near elimination, and diseases such as diphtheria, measles, mumps, pertussis, and tetanus are contained. Nonetheless, microbes remain major killers with current vaccines addressing only a handful of the infections of man and his domesticated animals. Common infectious diseases for which there are no vaccines cost the United States $120 billion dollars per year (Robinson et al., 1997). In first world countries, emerging infections such as immunodeficiency viruses, as well as reemerging diseases like drug resistant forms of tuberculosis, pose new threats and challenges for vaccine development. The need for both new and improved vaccines is even more pronounced in third world countries where effective vaccines are often unavailable or cost-prohibitive. Recently, direct injections of antigen-expressing DNAs have been shown to initiate protective immune responses.

DNA-based vaccines use bacterial plasmids to express protein immunogens in vaccinated hosts. Recombinant DNA technology is used to clone cDNAs encoding immunogens of interest into eukaryotic expression plasmids. Vaccine plasmids are then amplified in bacteria, purified, and directly inoculated into the hosts being vaccinated. DNA typically is inoculated by a needle injection

of DNA in saline, or by a gene gun device that delivers DNA-coated gold beads into skin. The plasmid DNA is taken up by host cells, the vaccine protein is expressed, processed and presented in the context of self-major histocompatibility (MHC) class I and class II molecules, and an immune response against the DNA-encoded immunogen is generated.

The historical foundations for DNA vaccines (also known as "genetic immunization") emerged concurrently from studies on gene therapy and studies using retroviral vectors. Gene therapy studies on DNA delivery into muscle revealed that pure DNA was as effective as liposome-encapsulated DNA at mediating transfection of skeletal muscle cells (Wolff et al., 1990). This unencapsulated DNA was termed "naked DNA," a fanciful term that has become popular for the description of the pure DNA used for nucleic acid vaccinations. Gene guns, which had been developed to deliver DNA into plant cells, were also used in gene therapy studies to deliver DNA into skin. In a series of experiments testing the ability of plasmid-expressed human growth hormone to alter the growth of mice, it was realized that the plasmid inoculations, which had failed to alter growth, had elicited antibody (Tang, De Vit, and Johnston, 1992). This was the first demonstration of the raising of an immune response by an inoculated plasmid DNA. At the same time, experiments using retroviral vectors, demonstrated that protective immune responses could be raised by very few infected cells (on the order of 10^4 - 10^5). Direct tests of the plasmid DNA that had been used to produce infectious forms of the retroviral vector for vaccination, performed in an influenza model in chickens, resulted in protective

immunizations (Robinson, Hunt, and Webster, 1993).

HIV-1 is projected to infect 1% of the world's population by the year 2000, making vaccine development for this recently emergent agent a high priority for world health. Preclinical trials on DNA vaccines have demonstrated that DNA alone can protect against highly attenuated HIV-1 challenges in chimpanzees (Boyer et al., 1997), but not against more virulent SIV challenges in macaques (Lu et al., 1997). A combination of DNA priming plus an envelope glycoprotein boost has raised a neutralizing antibody-associated protection against a homologous challenge with a non-pathogenic chimera between SIV and HIV (SHIV-IIIb) (Letvin et al., 1997). More recently, a comparative trial testing eight different protocols for the ability to protect against a series of challenges with SHIV-s (chimeras between simian and human immunodeficiency viruses) revealed the best containment of challenge infections by an immunization protocol that included priming by intradermal inoculation of DNA and boosting with recombinant fowl pox virus vectors (Robinson et al., 1999). This containment of challenge infections was independent of the presence of neutralizing antibody to the challenge virus. Protocols which proved less effective at containing challenge infections included immunization by both priming and boosting by intradermal or gene gun DNA inoculations, immunization by priming with intradermal or gene gun DNA inoculations and then boosting with a protein subunit; immunization by priming with gene gun DNA inoculations and boosting with recombinant fowl pox virus, immunization with protein only, and immunization with recombinant fowl pox virus only

(Robinson et al,1999). Early clinical trials of DNA vaccines in humans have revealed no adverse effects (MacGregor et al., 1996) and the raising of cytolytic T-cells (Calarota et al., 1998). A number of studies have screened for the ability of co-transfected lymphokines and co-stimulatory molecules to increase the efficiency of immunization (Robinson and Pertmer, in press).

Disadvantages of DNA vaccine approaches include the limitation of immunizations to products encoded by DNA (e.g., proteins) and the potential for atypical processing of bacterial and parasitic proteins by eukaryotic cells. Another significant problem with existing approaches to DNA vaccines is the instability of some vaccine insert sequences during the growth and amplification of DNA vaccine plasmids in bacteria. One possible cause of instability is exposure during plasmid growth of secondary structures in vaccine inserts or the plasmid backbone that can be recognized by bacterial endonucleases.

A need exists, therefore, for DNA expression vectors that exhibit improved stability in bacterial hosts and may be safely used in animals, including humans, for eukaryotic expression of immunogenic proteins useful as vaccines against a variety of infectious diseases, including HIV-1.

SUMMARY OF THE INVENTION

The present invention provides novel pGA constructs. The novel pGA constructs are useful as vectors for the delivery of DNA vaccines.

The present invention also provides novel pGA constructs having vaccine inserts. The pathogen vaccine inserts can include the DNA transcription unit of any virus, bacteria, parasite and/or fungi.

The present invention describes novel methods of immunizing patients by administering therapeutically effective amounts of the novel pGA constructs comprising pathogen vaccine inserts.

The present invention describes novel methods of immunizing patients by administering therapeutically effective amounts of the novel pGA constructs comprising pathogen vaccine inserts followed by booster immunizations with live vectored vaccines such as recombinant modified vaccinia Ankara (MVA) vectors comprising the same vaccine inserts.

The present invention also describes novel methods of raising mult-epitope CD8 T-cell responses by administering therapeutically effective amounts of the novel pGA constructs comprising pathogen vaccine inserts followed by booster immunizations with a live vectored vaccine such as recombinant modified vaccinia Ankara (MVA) vectors comprising the same vaccine inserts.

The present invention is described in more detail below.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates a novel pGA1 construct of the present invention. Designations are identities and positions of elements in the vector. Designations in italic print are unique restriction endonuclease sites useful for cloning vaccine inserts into the vector.

Fig. 2 illustrates the DNA sequence SEQ ID NO: 1 of the novel pGA1 construct shown in **Fig. 1**. The positions of elements in the plasmid are indicated below the nucleotide sequence.

Fig. 3 illustrates a novel pGA2 construct of the present invention. Designations are identities and positions of elements in the vector. Designations in italic print are unique restriction endonuclease sites useful for cloning vaccine inserts into the vector.

Fig. 4 illustrates the DNA sequence SEQ ID NO: 2 of the novel pGA2 construct shown in **Fig. 3**. The positions of elements in the plasmid are indicated below the nucleotide sequence. **Fig. 5** illustrates a novel pGA3 construct of the present invention. Designations are identities and positions of elements in the vector. Designations in italic print are unique restriction endonuclease sites useful for cloning vaccine inserts into the vector.

Fig. 6 illustrates the DNA sequence SEQ ID NO: 3 of the novel pGA3 construct shown in **Fig. 5**. The positions of elements in the plasmid are indicated below the nucleotide sequence.

Fig. 7 compares the levels of anti-HA IgG raised by the influenza H1 hemagglutinin expressed in a pGA vector (pGA3/H1) and in the pJW4303 research vector (pJW4303/H1). BALB/c mice were immunized and boosted with a low dose (0.1 μg) or a high dose (1 μg), of the indicated plasmids using gene gun inoculations. A priming immunization was followed by a booster immunization at 4 weeks.

Fig. 8A presents a schematic of the parent wt BH10 provirus from which constructs producing non-infectious virus like particles (VLPs) were produced. Dotted regions indicate sequences that were deleted in the VLP constructs. Positions and designations of the various regions of the BH10 provirus are

indicated in the rectangular boxes. The U3RU5 regions which encode the long terminal repeats contain transcriptional control elements. All other indicated regions encode proteins. For clarity, products expressed by *pol* (Prt, RT, Int) and *env* (SU and TM) are indicated.

Fig. 8B depicts the JS2 vaccine insert. This 6.7 kb vaccine insert expresses the Gag, Prt, and RT sequences of the BH10 strain of HIV-1-IIIb, Tat and Vpu proteins that are from ADA, and Rev and Env proteins that are chimeras of ADA and BH10 sequences. The Gag sequences include mutations of the zinc fingers to limit packaging of viral RNA. The RT sequences encompass three point mutations to eliminate reverse transcriptase activity. Designations are the same as in Fig. 8A. The bracketed area indicates the region of BH10 in which sequences from ADA have been substituted for the BH10 sequences to introduce a CCR-5 using Env. The x's indicate safety mutations.

Fig. 8C depicts the JS5 insert. JS5 is a 6kb vaccine insert that expresses Gag, Prt, RT, Vpu Tat, and Rev. JS5 is comprised of the same sequences as JS2 except that sequences in Env have been deleted. The deleted sequences are indicated in Figure 8B as a filled rectangle. Designations are the same as in Figs. 8A and 8B. The Rev responsive element (RRE) which is in the 3' region of Env is retained in the construct.

Figs. 9A and **9B** show Gag and Env expression, respectively, for intermediates in the construction of the JS2 vaccine insert. Data are from transient transfections in 293T cells. pGA1/JS1 (ADA VLP) produced higher levels of both Gag (**Fig. 9A**) and Env (**Fig. 9B**) than wild type HIV-1 ADA or

HIV-1 IIIb proviruses, and a VLP-producing DNA (dPol) used in previous studies.

Fig. 10 shows the expression of p24 capsid in transiently transfected cells by vaccine vectors expressing inserts without safety mutations (JS1 and JS4), inserts with point mutations in the zinc fingers and in RT (JS2 and JS5), and point mutations in the zinc fingers, RT, and protease (JS3 and JS6). Note that the safety mutations in the zinc fingers and RT supported active VLP expression whereas the safety mutation in Prt did not. JS2 and JS5 were chosen for continued vector development based on their high levels of expression in the presence of safety mutations.

Figs. 11A and 11B show Gag and Env expression, respectively, of novel candidate vaccine constructs expressed by pGA vectors with and without intron A. PGA1 but not pGA2 contains intron A. pGA2/JS2 and pGA1/JS5 were chosen for use in vaccines based on their favorable levels of expression.

Figs. 12A-12D shows Western blots of cell lysates and tissue culture supernatants from 293T cells transfected with (1) mock, (2) pGA2/JS2, and (3) pGA1/JS5, where the primary antibody was pooled from anti-HIV Ig from infected patients **(Fig. 12A)**, anti-p24 **(Fig. 12B)**, anti-gp120 **(Fig. 12C)**, and anti-RT **(Fig. 12D)** respectively.

Fig. 13 illustrates pGA.

Fig. 14 compares Gag expression levels between pGA2/89.6, pGA1/Gag-Pol and pGA2/JS2. Comparative studies for expression were performed on transiently transfected 293T cells.

Figs. 15A-15C show the temporal frequencies of Gag-specific T cells. **Fig. 15A:** Gag-specific CD8 T Cell responses raised by DNA priming and rMVA booster immunization. The schematic presents Gag-CM9-tetramer data generated in the high-dose i.d. DNA-immunized animals. **Fig. 15B**: Gag-CM9-Mamu-A*01 tetramer- specific T cells in *Mamu-A*01* vaccinated and control macaques at various times before challenge and at two weeks after challenge. The number at the upper right corner of each plot represents the frequency of tetramer-specific CD8 T cells as a % of total CD8 T cells. The numbers above each column of plots designate individual animals. **Fig. 15C:** Gag–specific IFN-γ ELISPOTs in *A*01* and non- *A*01* (hatched bars) vaccinated and non-vaccinated macaques at various times before challenge and at two weeks after challenge. Three pools of approximately 10-13 Gag peptides (22-mers overlapping by 12) were used for the analyses. The numbers above data bars represent the arithmetic mean ± the standard deviation for the ELISPOTs within each group. The numbers at the top of the graphs designate individual animals. *, data not available; #, <20 ELISPOTs per $1x10^6$ PBMC.

Figs. 16A-16B shows the height and breadth of IFN-γ-producing ELISPOTs against Gag and Env in the DNA/MVA memory response. **Fig. 16A:** Responses against individual Gag and Env peptide pools. Data for animals within a group are designated by the same symbol. **Fig. 16B:** Averages of the height and breadth of ELISPOT responses for the different groups. The heights are the mean ± the standard deviation for the sums of the Gag and Env ELISPOTs for animals in each group. The breadths are the mean ± the standard deviation for the number

of Gag and Env pools recognized by animals in each group. ELISPOT responses were determined in PBMC, during the memory phase, at 25 weeks after the rMVA booster (four weeks prior to challenge) using 7 pools of Gag peptides (approximately seven 22-mers overlapping by 12) representing about 70 amino acids of Gag sequence, and 21 pools of Env peptides (approximately ten 15-mers overlapping by 11) representing about 40 amino acids of Env sequence.

Fig. 17 shows the DNA sequence SEQ ID NO: 4 of a pGA2 construct comprising the vaccine insert, where the pathogen vaccine insert. JS2 expresses clade B HIV-1 VLP. Both the nucleotide sequence SEQID NO: 4 and encoded proteins are indicated.

Fig. 18 shows the DNA sequence of a pGA1 construct comprising a pathogen vaccine insert, where the pathogen vaccine insert. JS5 expresses clade B HIV-1 Gag-pol insert. Both the sequence and the encoded proteins are shown.

Figs. 19A-19E show temporal viral loads, CD4 counts and survival after challenge of vaccinated and control animals. **Fig. 19A:** Geometric mean viral loads and **Fig. 19B:** geometric mean CD4 counts for vaccine and control groups at various weeks post-challenge. The key for the groups is in panel B. **Fig. 19C:** Survival curve for vaccinated and control animals. The dotted line represents all 24 vaccinated animals. **Fig. 19D:** viral loads and **Fig. 19E:** CD4 counts for individual animals in the vaccine and control groups. The key to animal numbers is presented in **Fig. 19E**. Assays for the first 12 weeks post challenge had a background of 1000 copies of RNA per ml of plasma. Animals with loads below 1000 were scored with a load of 500. For weeks 16 and 20, the background for

detection was 300 copies of RNA/ml. Animals with levels of virus below 300 were scored at 300.

Figs. 20A-20C show Post-challenge T-cell responses in vaccine and control groups. **Fig. 20A:** temporal tetramer+ cells and viral loads. **Fig. 20B:** Intracellular cytokine assays for IFN-γ production in response to stimulation with the Gag-CM9 peptide at two weeks post-challenge. This *ex vivo* assay allows evaluation of the functional status of the peak post-challenge tetramer+ cells displayed in Figure 15A. **Fig. 20C:** Proliferation assay at 12 weeks post-challenge. Gag-Pol-Env (open bars) and Gag-Pol (hatched bars) produced by transient transfections were used for stimulation. Supernatants from mock-transfected cultures served as control antigen. Proteins were used at approximately 1 μg per ml of p27 Gag for stimulations. Stimulation indices are the growth of cultures in the presence of viral antigens divided by the growth of cultures in the presence of mock antigen.

Figs. 21A-21E show lymph node histomorphology and viral loads at 12 weeks post-challenge. **Fig. 21A:** Typical lymph node from a vaccinated macaque showing evidence of follicular hyperplasia characterized by the presence of numerous secondary follicles with expanded germinal centers and discrete dark and light zones. **Fig. 21B:** Typical lymph node from an infected control animal showing follicular depletion and paracortical lymphocellular atrophy. **Fig. 21C:** A representative lymph node from an age-matched, uninfected macaque displaying non-reactive germinal centers. **Fig. 21D:** The percent of the total lymph node area occupied by germinal centers was measured to give a non-

specific indicator of follicular hyperplasia. Data for uninfected controls are for four age-matched rhesus macaques. **Fig. 21E:** Lymph node virus burden was determined by in situ hybridization using an antisense riboprobe cocktail that was complementary to SHIV-89.6 *gag* and *pol*. All of the examined nodes were inguinal lymph nodes.

Figs. 22A-22D show temporal antibody responses following challenge. Micrograms of total Gag **(Fig. 22A)** or Env **(Fig. 22B)** antibody were determined using enzyme linked immunosorbent assays (ELISAs). The titers of neutralizing antibody for 89.6 **(Fig. 22C)** and 89.6P **(Fig. 22D)** were determined using MT-2 cell killing and neutral red staining. Titers are the reciprocal of the serum dilution giving 50% neutralization of the indicated viruses grown in human PBMC. Symbols for animals are the same as in Figure 19.

Figs. 23A-23E show correlations and dose response curves for the vaccine trial **(Figs. 23A and B)**. Inverse correlations between peak vaccine raised IFN-γ ELISPOTs and viral loads at 2 **(Fig. 23A)** and 3 **(Fig. 23B)** weeks post-challenge. Only twenty-three of the 24 vaccinated animals are included in the correlations because of the loss of the peak DNA/MVA ELISPOT sample for animal 3 (see Fig 15C). **(Fig. 23C)** Dose response curves for the average height of Gag ELISPOTS at the peak DNA-MVA response (data from Fig. 15C). **(Fig. 23D)** Dose response curve for the breadth of the DNA/MVA memory ELISPOT response (data from Fig. 16B). **(Fig. 23E)** Dose response curves for the peak anti-Gag antibody response post the MVA booster (data from Fig. 22A). The different doses of DNA raised different levels of ELISPOT and antibody

responses (P<0.05). The route of DNA inoculation had a significant effect on the antibody (P=0.02), but not the ELISPOT response.

Fig 24 shows anti-HA IgG raised by gene gun inoculation of DNAs expressing HA proteins.

Fig. 25. Shows avidity of the anti HA IgG raised by the three different HA DNA vaccines.

Fig. 26 shows protection from weight loss after virus challenge.

Fig. 27 illustrates the importance of including Env in the vaccine.

Figs. 28A-28D illustrates the importance of including Env in vaccines administered to animals challenged interectally with SHIV-89.6P.

Fig. 29 is a schematic representation of vector DNA vaccine constructs.

Fig. 30 shows Western blot results showing expression of vaccine constructs in vitro.

Fig. 31 is a temporal curve of measles virus neutralizing antibody.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to novel vectors, novel vectors comprising pathogen vaccine inserts, and novel methods of immunizing patients against a pathogen. The novel immunization methods elicit both cell-mediated and humoral immune responses that may limit the infection, spread or growth of the pathogen and result in protection against subsequent challenge by the pathogen.

Classic references for DNA vaccines include the first demonstration of the raising of an immune response (Tang, De Vit, and Johnston, 1992); the first demonstration of cytotoxic T-cell (Tc)-mediated immunity (Ulmer et al., 1993); the first demonstration of the protective efficacy of intradermal (i.d.), intramuscular (i.m.), intravenous (i.v.), intranasal (i.n.), and gene gun (g.g.) immunizations (Fynan et al., 1993; Robinson, Hunt, and Webster, 1993); the first use of genetic adjuvants (Xiang and Ertl, 1995); the first use of library immunizations (Barry, Lai, and Johnston, 1995); and the first demonstration of the ability to modulate the T-helper type of an immune response by the method of DNA delivery (Feltquate et al., 1997). A highly useful web site compiling DNA vaccine information can be found at http://www.genweb.com/Dnavax/dnavax.html.

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

Definitions

The term "nucleic acid" as used herein refers to any natural and synthetic linear and sequential arrays of nucleotides and nucleosides, for example cDNA,

genomic DNA, mRNA, tRNA, oligonucleotides, oligonucleosides and derivatives thereof. For ease of discussion, such nucleic acids may be collectively referred to herein as “constructs,” “plasmids,” or “vectors.” Representative examples of the nucleic acids of the present invention include bacterial plasmid vectors including expression, cloning, cosmid and transformation vectors such as, but not limited to, pBR322, animal viral vectors such as, but not limited to, modified adenovirus, influenza virus, polio virus, pox virus, retrovirus, and the like, vectors derived from bacteriophage nucleic acid, and synthetic oligonucleotides like chemically synthesized DNA or RNA. The term "nucleic acid" further includes modified or derivatised nucleotides and nucleosides such as, but not limited to, halogenated nucleotides such as, but not only, 5-bromouracil, and derivatised nucleotides such as biotin-labeled nucleotides.

The term “isolated nucleic acid” as used herein refers to a nucleic acid with a structure (a) not identical to that of any naturally occurring nucleic acid or (b) not identical to that of any fragment of a naturally occurring genomic nucleic acid spanning more than three separate genes, and includes DNA, RNA, or derivatives or variants thereof. The term includes, but is not limited to, the following: (a) a DNA which has the sequence of part of a naturally occurring genomic molecule but is not flanked by at least one of the coding sequences that flank that part of the molecule in the genome of the species in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic nucleic acid of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any vector or naturally occurring genomic DNA; (c) a separate

molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), ligase chain reaction (LCR) or chemical synthesis, or a restriction fragment; (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein, and (e) a recombinant nucleotide sequence that is part of a hybrid sequence that is not naturally occurring.

It is advantageous for some purposes that a nucleotide sequence is in purified form. The term "purified" in reference to nucleic acid represents that the sequence has increased purity relative to the natural environment.

As used herein the terms "polypeptide" and "protein" refer to a polymer of amino acids of three or more amino acids in a serial array, linked through peptide bonds. The term "polypeptide" includes proteins, protein fragments, protein analogues, oligopeptides and the like. The term "polypeptides" contemplates polypeptides as defined above that are encoded by nucleic acids, produced through recombinant technology, isolated from an appropriate source, or are synthesized. The term "polypeptides" further contemplates polypeptides as defined above that include chemically modified amino acids or amino acids covalently or noncovalently linked to labeling ligands.

The term "fragment" as used herein to refer to a nucleic acid (e.g., cDNA) refers to an isolated portion of the subject nucleic acid constructed artificially (e.g., by chemical synthesis) or by cleaving a natural product into multiple pieces, using restriction endonucleases or mechanical shearing, or a portion of a nucleic acid synthesized by PCR, DNA polymerase or any other polymerizing technique

well known in the art, or expressed in a host cell by recombinant nucleic acid technology well known to one of skill in the art. The term "fragment" as used herein may also refer to an isolated portion of a polypeptide, wherein the portion of the polypeptide is cleaved from a naturally occurring polypeptide by proteolytic cleavage by at least one protease, or is a portion of the naturally occurring polypeptide synthesized by chemical methods well known to one of skill in the art.

The term “gene” or “genes” as used herein refers to nucleic acid sequences (including both RNA or DNA) that encode genetic information for the synthesis of a whole RNA, a whole protein, or any portion of such whole RNA or whole protein. Genes that are not naturally part of a particular organism’s genome are referred to as “foreign genes”, "heterologous genes" or "exogenous genes" and genes that are naturally a part of a particular organism’s genome are referred to as “endogenous genes”.

The term “expressed” or “expression” as used herein refers to the transcription from a gene to give an RNA nucleic acid molecule at least complementary in part to a region of one of the two nucleic acid strands of the gene. The term “expressed” or “expression” as used herein also refers to the translation from said RNA nucleic acid molecule to give a protein or polypeptide or a portion thereof.

As used herein, the term “locus” or “loci” refers to the site of a gene on a chromosome. Pairs of genes control hereditary traits, each in the same position on a pair of chromosomes. These gene pairs, or alleles, may both be dominant or

both be recessive in expression of that trait. In either case, the individual is said to be homozygous for the trait controlled by that gene pair. If the gene pair (alleles) consists of one dominant and one recessive trait, the individual is heterozygous for the trait controlled by the gene pair. Natural variation in genes or nucleic acid molecules caused by, for example, recombination events or resulting from mutation, gives rise to allelic variants with similar, but not identical, nucleotide sequences. Such allelic variants typically encode proteins with similar activity to that of the protein encoded by the gene to which they are compared, because natural selection typically selects against variations that alter function. Allelic variants can also comprise alterations in the untranslated regions of the gene as, for example, in the 3' or 5' untranslated regions or can involve alternate splicing of a nascent transcript, resulting in alternative exons being positioned adjacently.

The term "transcription regulatory sequences" as used herein refers to nucleotide sequences that are associated with a gene nucleic acid sequence and which regulate the transcriptional expression of the gene. The "transcription regulatory sequences" may be isolated and incorporated into a vector nucleic acid to enable regulated transcription in appropriate cells of portions of the vector DNA. The "transcription regulatory sequence" may precede, but are not limited to, the region of a nucleic acid sequence that is in the region 5' of the end of a protein coding sequence that may be transcribed into mRNA. Transcriptional regulatory sequences may also be located within a protein coding region, in regions of a gene that are identified as "intron" regions, or may be in regions of

nucleic acid sequence that are in the region of nucleic acid.

The term "coding region" as used herein refers to a continuous linear arrangement of nucleotides that may be translated into a protein. A full length coding region is translated into a full length protein; that is, a complete protein as would be translated in its natural state absent any post-translational modifications. A full length coding region may also include any leader protein sequence or any other region of the protein that may be excised naturally from the translated protein.

The term "probe" as used herein, when referring to a nucleic acid, refers to a nucleotide sequence that can be used to hybridize with and thereby identify the presence of a complementary sequence, or a complementary sequence differing from the probe sequence but not to a degree that prevents hybridization under the hybridization stringency conditions used. The probe may be modified with labels such as, but not only, radioactive groups, biotin, or any other label that is well known in the art.

The term "nucleic acid vector" as used herein refers to a natural or synthetic single or double stranded plasmid or viral nucleic acid molecule that can be transfected or transformed into cells and replicate independently of, or within, the host cell genome. A circular double stranded plasmid can be linearized by treatment with an appropriate restriction enzyme based on the nucleotide sequence of the plasmid vector. A nucleic acid can be inserted into a vector by cutting the vector with restriction enzymes and ligating the pieces together. The nucleic acid molecule can be RNA or DNA.

The term "expression vector" as used herein refers to a nucleic acid vector that may further include at least one regulatory sequence operably linked to a nucleotide sequence coding for the Mago protein. Regulatory sequences are well recognized in the art and may be selected to ensure good expression of the linked nucleotide sequence without undue experimentation by those skilled in the art. As used herein, the term "regulatory sequences" includes promoters, enhancers, and other elements that may control expression. Standard molecular biology textbooks such as *Sambrook et al.* eds "Molecular Cloning: A Laboratory Manual" 2nd ed. Cold Spring Harbor Press (1989) may be consulted to design suitable expression vectors, promoters, and other expression control elements. It should be recognized, however, that the choice of a suitable expression vector depends upon multiple factors including the choice of the host cell to be transformed and/or the type of protein to be expressed.

The terms "transformation" and "transfection" as used herein refer to the process of inserting a nucleic acid into a host. Many techniques are well known to those skilled in the art to facilitate transformation or transfection of a nucleic acid into a prokaryotic or eukaryotic organism. These methods involve a variety of techniques, such as treating the cells with high concentrations of salt such as, but not only a calcium or magnesium salt, an electric field, detergent, or liposome mediated transfection, to render the host cell competent for the uptake of the nucleic acid molecules.

The term "recombinant cell" refers to a cell that has a new combination of nucleic acid segments that are not covalently linked to each other in nature. A

new combination of nucleic acid segments can be introduced into an organism using a wide array of nucleic acid manipulation techniques available to those skilled in the art. A recombinant cell can be a single eukaryotic cell, or a single prokaryotic cell, or a mammalian cell. The recombinant cell can harbor a vector that is extragenomic. An extragenomic nucleic acid vector does not insert into the cell's genome. A recombinant cell can further harbor a vector or a portion thereof that is intragenomic. The term intragenomic defines a nucleic acid construct incorporated within the recombinant cell's genome.

The term "recombinant nucleic acid" as used herein refers to combinations of at least two nucleic acid sequences that are not naturally found in a eukaryotic or prokaryotic cell. The nucleic acid sequences may include, but are not limited to nucleic acid vectors, gene expression regulatory elements, origins of replication, sequences that when expressed confer antibiotic resistance, and protein-encoding sequences. The term "recombinant polypeptide" is meant to include a polypeptide produced by recombinant DNA techniques such that it is distinct from a naturally occurring polypeptide either in its location, purity or structure. Generally, such a recombinant polypeptide will be present in a cell in an amount different from that normally observed in nature.

The term "patients," as used herein, refers to animals, preferably mammals, and more preferably humans.

The term "immunizing" or "immunization," as used herein, refers to the production of an immune response in a patient that protects (partially or totally) from the manifestations of infection (i.e., disease) caused by a pathogen. A

patient immunized by the present invention will not be infected by the pathogen or will be infected to a lesser extent than would occur without immunization. Immunizations may be either prophylactic or therapeutic in nature. That is, both previously uninfected and infected patients may be immunized with the present invention.

The term "DNA transcription unit" as used herein" refers to a polynucleotide sequence that includes at least two components: antigen-encoding DNA and transcriptional promoter elements. A DNA transcription unit may optionally include additional sequences, such as enhancer elements, splicing signals, termination and polyadenylation signals, viral replicons, and/or bacterial plasmid sequences. The DNA transcription unit can be produced by a number of known methods. For example, DNA encoding the desired antigen can be inserted into an expression vector to construct the DNA transcription unit, as described in Maniatis et al, *Molecular Cloning: A Laboratory Manual*, 2d, Cold Spring Harbor Laboratory Press (1989), the disclosure of which is incorporated by reference in its entirety.

The term "vaccine insert" as used herein refers to the DNA transcription unit of a pathogen. Preferably, the vaccine insert is a DNA transcription unit that can generate an immune responses in a patient. For example, th evaccine insert is a pathogen vaccine insert encoding antigens derived from any virus, bacteria, parasite and/or fungi. Exemplary viruses include herpesvirus, orthomyxoviruses, rhinoviruses, picornaviruses, adenoviruses, paramyxoviruses, coronaviruses, rhabdoviruses, togaviruses, flaviviruses, bunyaviruses, rubella virus, reovirus,

measles, hepadna viruses, Ebola, retroviruses (including human immunodeficiency virus), and the like. Exemplary bacteria include tuberculosis, mycobateria, spirochetes, rickettsias, chlamydia, mycoplasma and the like. Exemplary parasites include malaria and the like. Exemplary fungi include yeasts, molds, and the like. One skilled in the art will appreciate that this list does not include all potential pathogens against which a protective immune response can be generated by the methods described herein.

The term "antigen" as used herein refers to any protein, carbohydrate, or other moiety expressed by a pathogen that is capable of eliciting a protective response against a pathogen. The antigen may or may not be a structural component of the pathogen. Also contemplated to be within the term "antigen" are encoded antigens that can be translation products or polypeptides of various lengths. Antigens undergo normal host cell modifications such as glycosylation, myristoylation or phosphorylation. In addition, they can be designed to undergo intracellular, extracellular or cell-surface expression. Furthermore, they can be designed to undergo assembly and release from cells.

As used herein, the term "adjuvant" means a substance added to a vaccine to increase a vaccine's immunogenicity. The mechanism of how an adjuvant operates is not entirely known. Some adjuvants are believed to enhance the immune response by slowly releasing the antigen, while other adjuvants are strongly immunogenic in their own right and are believed to function synergistically. Known vaccine adjuvants include, but are not limited to, oil and water emulsions (for example, complete Freund's adjuvant and incomplete

Freund's adjuvant), *Corynebacterium parvum*, Bacillus Calmette Guerin, aluminum hydroxide, glucan, dextran sulfate, iron oxide, sodium alginate, Bacto-Adjuvant, certain synthetic polymers such as poly amino acids and co-polymers of amino acids, saponin, "REGRESSIN" (Vetrepharm, Athens, Ga.), "AVRIDINE" (N, N-dioctadecyl-N',N'-bis(2-hydroxyethyl)-propanediamine), paraffin oil, and muramyl dipeptide. Adjuvants also encompass genetic adjuvants such as immunomodulatory molecules encoded in a co-inoculated DNA. The co-inoculated DNA can be in the same vaccine construct as the vaccine immunogen or in a separate DNA vector.

As used herein, the term "pharmaceutically acceptable carrier" means a vehicle for containing the vaccine that can be injected into a bovine host without adverse effects. Suitable pharmaceutically acceptable carriers known in the art include, but are not limited to, sterile water, saline, glucose, dextrose, or buffered solutions. Carriers may include auxiliary agents including, but not limited to, diluents, stabilizers (i.e., sugars and amino acids), preservatives, wetting agents, emulsifying agents, pH buffering agents, viscosity enhancing additives, colors and the like.

The terms "selectable marker gene" as used herein refer to an expressed gene that allows for the selection of a population of cells containing the selectable marker gene from a population of cells not having the expressed selectable marker gene. For example, the "selectable marker gene" may be an "antibiotic resistance gene" that can confer tolerance to a specific antibiotic by a microorganism that was previously adversely affected by the drug. Such resistance may result from a

mutation or the acquisition of resistance due to plasmids containing the resistance gene transforming the microorganism.

The term "terminator sequence" or "terminator" as used herein refers to nucleotide sequences that function to stop transcription. The terms "transcription" or "transcribe" as used herein refers to the process by which RNA molecules are formed upon DNA templates by complementary base pairing. This process is mediated by RNA polymerase.

The term "VLP" as used herein refers to virus-like particles and, as used, also refers to aggregates of viral proteins.

The major immunological advantage of DNA-based immunizations is the ability of the immunogen to be presented by both MHC class I and class II molecules. Endogenously synthesized proteins readily enter processing pathways for the loading of peptide epitopes onto MHC I as well as MHC II molecules. MHC I-presented epitopes raise cytotoxic T-cells (Tc) responses whereas MHC II-presented epitopes raise helper T-cells (Th). By contrast, immunogens that are not synthesized in cells are largely restricted to the loading of MHC II epitopes and the raising of Th but not Tc. When compared with live attenuated vaccines or recombinant viral vectors that produce immunogens in cells and raise both Th and Tc, DNA vaccines have the advantages of not being infectious and of focusing the immune response on only those antigens desired for immunization. DNA vaccines also are advantageous because they can be manipulated relatively easily to raise type 1 or type 2 T-cell help. This allows a vaccine to be tailored for the type of immune response that will be mobilized to combat an infection. DNA

vaccines are also cost effective because of the ease with which plasmids can be constructed using recombinant DNA technology, the ability to use a generic method for vaccine production (growth and purification of plasmid DNA), and the stability of DNA over a wide range of temperatures.

The best immune responses are achieved using highly active expression vectors modeled on those developed for the production of recombinant proteins (Robinson and Pertmer, 1998). The most frequently used transcriptional control elements include a strong promoter. One such promoter suitable for use is the cytomegalovirus (CMV) intermediate early promoter, although other promoters may be used in a DNA vaccine without departing from the scope the present invention. Other transcriptional control elements useful in the present invention include a strong polyadenylation signal such as, for example, that derived from a bovine growth hormone encoding gene, or a rabbit β globin polyadenylation signal (Bohm et al., 1996; Chapman et al., 1991; Hartikka et al., 1996; Manthorpe et al., 1993; Montgomery et al., 1993). The CMV immediate early promoter may be used with or without intron A (Chapman et al., 1991). The presence of intron A increases the expression of many antigens from RNA viruses, bacteria, and parasites, presumably by providing the expressed RNA with sequences which support processing and function as an eukaryotic mRNA. It will be appreciated that expression also may be enhanced by other methods known in the art including, but not limited to, optimizing the codon usage of prokaryotic mRNAs for eukaryotic cells (Andre et al., 1998; Uchijima et al., 1998). Multi-cistronic vectors may be used to express more than one immunogen or an immunogen and

a immunostimulatory protein (Iwasaki et al., 1997a; Wild et al., 1998).

Immunogens can also be engineered to be more or less effective for raising antibody or Tc by targeting the expressed antigen to specific cellular compartments. For example, antibody responses are raised more effectively by antigens that are displayed on the plasma membrane of cells, or secreted therefrom, than by antigens that are localized to the interior of cells (Boyle, Koniaras, and Lew, 1997; Inchauspe et al., 1997). Tc responses may be enhanced by using N-terminal ubiquitination signals which target the DNA-encoded protein to the proteosome causing rapid cytoplasmic degradation and more efficient peptide loading into the MHC I pathway (Rodriguez, Zhang, and Whitton, 1997; Tobery and Siliciano, 1997; Wu and Kipps, 1997). For a review on the mechanistic basis for DNA-raised immune responses, refer to Robinson and Pertmer, *Advances in Virus Research*, vol. 53, Academic Press (2000), the disclosure of which is incorporated herein by reference in its entirety.

The effects of different conformational forms of proteins on antibody responses, the ability of strings of MHC I epitopes (minigenes) to raise Tc responses, and the effect of fusing an antigen with immune-targeting proteins have been evaluated using defined inserts. Ordered structures such as virus-like particles appear to be more effective than unordered structures at raising antibody (Fomsgaard et al., 1998). This is likely to reflect the regular array of an immunogen being more effective than a monomer of an antigen at cross-linking Ig-receptors and signaling a B-cell to multiply and produce antibody. Recombinant DNA molecules encoding a string of MHC epitopes from different

pathogens can elicit Tc responses to a number of pathogens (Hanke et al., 1998b). These strings of Tc epitopes are most effective if they also include a Th epitope (Maecker et al., 1998; Thomson et al., 1998).

Another approach to manipulating immune responses is to fuse immunogens to immunotargeting or immunostimulatory molecules. To date, the most successful of these fusions have targeted secreted immunogens to antigen presenting cells (APC) or lymph nodes (Boyle, Brady, and Lew, 1998). Fusion of a secreted form of human IgG with CTLA-4 increased antibody responses to the IgG greater than 1000-fold and changed the bias of the response from complement (C'-)dependent to C'-independent antibodies.

Fusions of human IgG with L-selectin also increased antibody responses but did not change the C'-binding characteristics of the raised antibody. The immunogen fused with L-selectin was presumably delivered to lymph nodes by binding to the high endothelial venules, which serve as portals. Fusions between antigens and cytokine cDNAs have resulted in more moderate increases in antibody, Th, and Tc responses (Hakim, Levy, and Levy, 1996; Maecker et al., 1997). IL-4-fusions have increased antibody responses, whereas IL-12 and IL-1β have enhanced T-cell responses.

Two approaches to DNA delivery are injection of DNA in saline using a hypodermic needle or gene gun delivery of DNA-coated gold beads. Saline injections deliver DNA into extracellular spaces, whereas gene gun deliveries bombard DNA directly into cells. The saline injections require much larger amounts of DNA (100-1000 times more) than the gene gun (Fynan et al., 1993).

These two types of delivery also differ in that saline injections bias responses towards type 1 T-cell help, whereas gene gun deliveries bias responses towards type 2 T-cell help (Feltquate et al., 1997; Pertmer, Roberts, and Haynes, 1996). DNAs injected in saline rapidly spread throughout the body. DNAs delivered by the gun are more localized at the target site. Following either method of inoculation, extracellular plasmid DNA has a short half life on the order of 10 minutes (Kawabata, Takakura, and Hashida, 1995; Lew et al., 1995). Vaccination by saline injections can be intramuscular (i.m.) or intradermal (i.d.) (Fynan et al., 1993).

Although intravenous and subcutaneous injections have met with different degrees of success for different plasmids (Bohm et al., 1998; Fynan et al., 1993), intraperitoneal injections have not met with success (Bohm et al., 1998; Fynan et al., 1993). Gene gun deliveries can be administered to the skin or to surgically exposed muscle. Methods and routes of DNA delivery that are effective at raising immune responses in mice are effective in other species.

Immunization by mucosal delivery of DNA has been less successful than immunizations using parenteral routes of inoculation. Intranasal administration of DNA in saline has met with both good (Asakura et al., 1997; Sasaki et al., 1998b) and limited (Fynan et al., 1993) success. The gene gun has successfully raised IgG following the delivery of DNA to the vaginal mucosa (Livingston et al., 1995). Some success at delivering DNA to mucosal surfaces has also been achieved using liposomes (McCluskie et al., 1998), microspheres (Chen et al., 1998a; Jones et al., 1997) and recombinant Shigella vectors (Sizemore,

Branstrom, and Sadoff, 1995; Sizemore, Branstrom, and Sadoff, 1997).

The dose of DNA needed to raise a response depends upon the method of delivery, the host, the vector, and the encoded antigen. The most profound effect is seen for the method of delivery. From 10 μg to 1 mg of DNA is generally used for saline injections of DNA, whereas from 0.2 μg to 20 μg of DNA is used for gene gun deliveries of DNA. In general, lower doses of DNA are used in mice (10-100 μg for saline injections and 0.2 μg to 2 μg for gene gun deliveries), and higher doses in primates (100 μg to 1 mg for saline injections and 2 μg to 20 μg for gene gun deliveries). The much lower amount of DNA required for gene gun deliveries reflect the gold beads directly delivering DNA into cells.

An example of the marked effect of an antigen on the raised response can be found in studies comparing the ability to raise antibody responses in rabbits of DNAs expressing the influenza hemagglutinin or an immunodeficiency virus envelope glycoprotein (Env) (Richmond et al., 1998). Under similar immunization conditions, the hemagglutinin-expressing DNA raised long lasting, high avidity, high titer antibody (~100 μg per ml of specific antibody), whereas the Env-expressing DNA raised only transient, low avidity, and low titer antibody responses (<10 μg per ml of specific antibody). These differences in raised antibody were hypothesized to reflect the hemagglutinin being a T-dependent antigen and the highly glycosylated immunodeficiency virus Env behaving as a T-independent antigen.

Both protein and recombinant viruses have been used to boost

DNA-primed immune responses. Protein boosts have been used to increase neutralizing antibody responses to the HIV-1 Env. Recombinant pox virus boosts have been used to increase both humoral and cellular immune responses.

For weak immunogens, such as the immunodeficiency virus Env, for which DNA-raised antibody responses are only a fraction of those in naturally infected animals, protein boosts have provided a means of increasing low titer antibody responses (Letvin et al., 1997; Richmond et al., 1998). In a study in rabbits, the protein boost increased both the titers of antibody and the avidity and the persistence of the antibody response (Richmond et al., 1998). Consistent with a secondary immune response to the protein boost, DNA primed animals showed both more rapid increases in antibody, and higher titers of antibody following a protein boost than animals receiving only the protein. However, by a second protein immunization, the kinetics and the titer of the antibody response were similar in animals that had, and had not, received DNA priming immunizations.

Recombinant pox virus boosts have proved to be a highly successful method of boosting DNA-primed CD8+ cell responses (Hanke et al., 1998a; Kent et al., 1998; Schneider et al., 1998). Following pox virus boosters, antigen-specific CD8+ cells have been increased by as much as 10-fold in DNA primed mice or macaques. Studies testing the order of immunizations reveal that the DNA must be delivered first (Schneider et al., 1998). This has been hypothesized to reflect the DNA focusing the immune response on the desired immunogens. The larger increases in CD8+ cell responses following pox virus boosts has been hypothesized to reflect both the larger amount of antigen

expressed by the pox virus vector, as well as pox virus-induced cytokines augmenting immune responses (Kent et al., 1998; Schneider et al., 1998).

A number of different pox viruses can be used for the pox boost. A vaccinia virus termed modified vaccinia Ankara (MVA) has been particularly effective in mouse models (Schneider et al., 1998). This may reflect MVA, which is replication defective in mammalian models, being attenuated for the ability to evade host immune responses.

Responses raised by a DNA prime followed by pox virus boost can be highly effective at raising protective cell-mediated immune responses. In mice, intramuscular injections of DNA followed by recombinant pox boosts have protected against a malaria challenge (Schneider et al., 1998). In macaques, intradermal., but not gene gun DNA primes, followed by recombinant pox virus boosters have contained challenges with chimeras of simian and human immunodeficiency viruses (Robinson et al., 1999).

DNA vaccines for immunodeficiency viruses such as HIV-1 encounter the challenge of sufficiently limiting an incoming infection such that the inexorable long- term infections that lead to AIDS are prevented. Complicating this is that neutralizing antibodies is both difficult to raise and specific against particular viral strains (Burton and Montefiori, 1997; Moore and Ho, 1995). Given the problems with raising neutralizing antibody, much effort has focused on raising cell-mediated responses of sufficient strength to severely curtail infections. To date, the best success at raising high titers of Tc have come from immunization protocols using DNA primes followed by recombinant pox virus boosters. The

efficacy of this protocol has been evaluated by determining the level of specific Tc using assays for cytolytic activity (Kent et al., 1998), by staining with MHC-specific tetramers for specific SIV Gag epitopes and by challenge with SIVs or SHIVs (Hanke, 1999).

A number of salient findings are emerging from preclinical trials using DNA primes and recombinant pox virus boosts. The first is that challenge infections can be contained below the level that can be detected using quantitative RT-PCR analyses for plasma viral RNA (Robinson et al., 1999). The second is that this protection is long lasting and does not require the presence of neutralizing antibody (Robinson et al., 1999). The third is that intradermal DNA priming with saline injections of DNA is superior to gene gun priming for raising protective immunity (P=0.01, Fisher's exact test) (Robinson et al., 1999).

The novel pGA vectors of the present invention have a prokaryotic origin of replication, a selective marker gene for plasmid selection, and a transcription cassette for eukaryotic cells. Unique to the pGA vectors of the present invention is the inclusion of the lambda terminator in the same transcriptional orientation, and following, the selective marker gene. This terminator sequence prevents read-through from the kanamycin cassette into vaccine sequences while the plasmid is being produced in bacteria. Prevention of transcriptional read-through stabilizes vaccine insert sequences by limiting the exposure of secondary structures that can be recognized by bacterial endonucleases.

A transcription cassette as incorporated in the pGA vectors of the present invention uses sequences from the cytomegalovirus immediate early promoter

(CMVIE) and from the bovine growth hormone polyadenylation sequences (BGHpA) to control transcription. A leader sequence that is a synthetic homolog of the tissue plasminogen activator gene leader sequence (tPA) is optional in the transcription cassette. The vectors of the present invention differ in the sites that can be used for accepting vaccine inserts and in whether the transcription cassette includes intron A sequences in the CMVIE promoter. Both intron A and the tPA leader sequence have been shown in certain instances to supply a strong expression advantage to vaccine inserts (Chapman et al., 1991).

pGA1 is a 3894 bp plasmid. pGA1 comprises a promoter (bp 1-690), the CMV-intron A (bp 691-1638), a synthetic mimic of the tPA leader sequence (bp 1659 – 1721), the bovine growth hormone polyadenylation sequence (bp1761-1983), the lambda T0 terminator (bp 1984-2018), the kanamycin resistance gene (bp 2037-2830) and the ColEI replicator (bp 2831-3890). The DNA sequence of the pGA1 construct (SEQ ID NO: 1) is shown in Fig. 2. In Fig. 1, the indicated restriction sites are single cutters useful for the cloning of vaccine inserts. The ClaI or BspD1 sites are used when the 5' end of a vaccine insert is cloned upstream of the tPA leader. The NheI site is used for cloning a sequence in frame with the tPA leader sequence. The sites listed between SmaI and BlnI are used for cloning the 3' terminus of a vaccine insert.

pGA2 is a 2947 bp plasmid lacking the 947 bp of intron A sequences found in pGA1. pGA2 is the same as pGA1, except for the deletion of intron A sequences. pGA2 is valuable for cloning sequences which do not require an upstream intron for efficient expression, or for cloning sequences in which an

upstream intron might interfere with the pattern of splicing needed for good expression. Fig. 3 presents a map of pGA2 with useful restriction sites for cloning vaccine inserts, and Fig. 4 shows the DNA sequence SEQ ID NO: 2. The use of restriction sites for cloning vaccine inserts into pGA2 is the same as that used for cloning fragments into pGA1.

pGA3 is a 3893 bp plasmid that contains intron A. pGA3 is the same as pGA1 except for the cloning sites that can be used for the introduction of vaccine inserts. In pGA3, inserts cloned upstream of the tPA leader sequence use a Hind III site. Sequences cloned downstream from the tPA leader sequence use sites between the SmaI and the BlnI site. In pGA3, these sites include a BamHI site. Fig. 5 shows the map for pGA3, and Fig. 6 shows the DNA sequence SEQ ID NO: 3.

In view of the teachings herein, one skilled in the art will recognize that any vaccine insert known in the art can be used in the novel pGA constructs described herein, including but not limited to viral pathogens like HIV, influenza, measles, herpes, Ebola, and the like.

For example, the present invention contemplates inserts from immunodeficiency virus, more preferably HIV, including all clades of HIV-1 and HIV-2 and modifications thereof; influenza virus genes including all subtypes and modifications thereof; and vaccine inserts derived from measles genes. One skilled in the art will appreciate that the discussion about inserts derived from immunodeficiency virus; influenza virus; measles virus; and modifications thereof are exemplary in nature and provided for the sake of illustration only.

The immunodeficiency virus vaccine inserts of the present invention were designed to express non-infectious virus like particles (VLPs) from a single DNA. This was achieved using the subgenomic splicing elements normally used by immunodeficiency viruses to express multiple gene products from a single viral RNA. Important to the subgenomic splicing patterns are (i) splice sites and acceptors present in full length viral RNA, (ii) the Rev responsive element (RRE) and (iii) the Rev protein. The splice sites in retroviral RNAs use the canonical sequences for splice sites in eukaryotic RNAs. The RRE is an ~200bp RNA structure that interacts with the Rev protein to allow transport of viral RNAs from the nucleus to the cytoplasm. In the absence of Rev, the ~10 kb RNA of immunodeficiency virus undergoes splicing to the mRNAs for the regulatory genes Tat, Rev, and Nef. These genes are encoded by exons present between RT and Env and at the 3' end of the genome. In the presence of Rev, the singly spliced mRNA for Env and the unspliced mRNA for Gag and Pol are expressed in addition to the multiply spliced mRNAs for Tat, Rev, and Nef.

The expression of non-infectious VLPs from a single DNA affords a number of advantageous features to an immunodeficiency virus vaccine. The expression of a number of proteins from a single DNA affords the vaccinated host the opportunity to respond to the breadth of T- and B-cell epitopes encompassed in these proteins. The expression of proteins containing multiple epitopes affords the opportunity for the presentation of epitopes by diverse histocompatibility types. By using whole proteins, one offers hosts of different histocompatibility types the opportunity to raise broad-based T-cell responses. Such may be

essential for the effective containment of immunodeficiency virus infections, whose high mutation rate supports ready escape from immune responses (Evans et al., 1999) (Poignard et al., 1999, Evans, *et al.*, 1995). Just as in drug therapy, multi-epitope T-cell responses that require multiple mutations for escape will provide better protection than single epitope T-cell responses that require only a single mutation for escape.

Antibody responses are often best primed by multi-valent vaccines that present an ordered array of an epitope to responding B-cells (Bachmann, Zinkernagel, 1997). Virus-like particles, by virtue of the multivalency of Env in the virion membrane, will facilitate the raising of anti-Env antibody responses. These particles will also present non-denatured and normal forms of Env to the immune system.

The novel vectors of the present invention can be administered to a patient in the presence of adjuvants or other substances that have the capability of promoting DNA uptake or recruiting immune system cells to the site of the inoculation. Embodiments include combining the DNA vaccine with conventional adjuvants or genetic adjuvants. Conventional adjuvants, including reagents that favor the stability and uptake of the DNA, recruit immune system cells to the site of inoculation, or facilitate the immune activation of responding lymphoid cells, include but are not limited to oil and water emulsions (for example, complete Freund's adjuvant and incomplete Freund's adjuvant), *Corynebacterium parvum*, Bacillus Calmette Guerin, aluminum hydroxide, glucan, dextran sulfate, iron oxide, sodium alginate, Bacto-Adjuvant, certain

synthetic polymers such as poly amino acids and co-polymers of amino acids, saponin, "REGRESSIN" (Vetrepharm, Athens, Ga.), "AVRIDINE" (N, N-dioctadecyl-N',N'-bis(2-hydroxyethyl)-propanediamine), paraffin oil, and muramyl dipeptide. The present invention also contemplates the use of genetic adjuvants such as immunomodulatory molecules encoded in a co-inoculated DNA. The co-inoculated DNA can be in the same vaccine construct as the vaccine immunogen or in a separate DNA vector.

A vaccine according to the present invention can be administered in a variety of ways including through any parenteral or topical route. For example, an individual can be inoculated by intravenous, intraperitoneal, intradermal, subcutaneous or intramuscular methods. Inoculation can be, for example, with a hypodermic needle, needleless delivery devices such as those that propel a stream of liquid into the target site, or with the use of a gene gun that bombards DNA on gold beads into the target site. The vector comprising the pathogen vaccine insert can be administered to a mucosal surface by a variety of methods including intranasal administration, i.e., nose drops or inhalants, or intrarectal or intravaginal administration by solutions, gels, foams, or suppositories. Alternatively, the vector comprising the vaccine insert can be orally administered in the form of a tablet, capsule, chewable tablet, syrup, emulsion, or the like. In an alternate embodiment, vectors can be administered transdermally, by passive skin patches, iontophoretic means, and the like.

Any appropriate physiologically acceptable medium is suitable for introducing the vector comprising the pathogen vaccine insert into the patient.

For example, suitable pharmaceutically acceptable carriers known in the art include, but are not limited to, sterile water, saline, glucose, dextrose, or buffered solutions. Carriers may include auxiliary agents including, but not limited to, diluents, stabilizers (i.e., sugars and amino acids), preservatives, wetting agents, emulsifying agents, pH buffering agents, viscosity enhancing additives, colors and the like.

The present invention is further illustrated by the following examples, which are provided by way of illustration and should not be construed as limiting. The contents of all references, published patents and patents cited throughout the present application are hereby incorporated by reference in their entirety.

Example 1: Structure and Sequence of pGA1

pGA1 as illustrated in Fig. 1 and Fig. 2 contains the ColE1 origin of replication, the kanamycin resistance gene for antibiotic selection, the lambda T0 terminator, and a eukaryotic expression cassette including an upstream intron. The ColE1 origin of replication is a 600 nucleotide DNA fragment that contains the origin of replication (*ori*), encodes an RNA primer, and encodes two negative regulators of replication initiation. All enzymatic functions for replication of the plasmid are provided by the bacterial host. The original constructed plasmid that contained the ColE1 replicator was pBR322 (Bolivar, et al. 1977; Sutcliffe, et al. 1978).

The kanamycin resistance gene is an antibiotic resistance gene for plasmid selection in bacteria. The lambda T0 terminator prevents read through

from the kanamycin resistance gene into the vaccine transcription cassette during prokaryotic growth of the plasmid (Scholtissek and Grosse, 1987). By preventing read through into the vaccine expression cassette, the terminator helps stabilize plasmid inserts during growth in bacteria.

The eukaryotic expression cassette is comprised of the CMV immediate early promoter including intron A (CMVIE-IA) and termination sequences from the bovine growth hormone polyadenylation sequence (BGHpA). A synthetic mimic of the leader sequence for tissue plasminogen activator (tPA) is included as an option within the transcription cassette. Cassettes with these elements have proven to be highly effective for expressing foreign genes in eukaryotic cells (Chapman et al., 1991). Cloning sites within the transcription cassette include a ClaI site upstream of the tPA leader, a NheI site for cloning in frame with the tPA leader, and XmnI, SmaI, RsrII, AvrII, and BlnI sites for cloning prior to the BGHpA.

The ColE1 replicator, the Kanamycin resistance gene and transcriptional control elements for eukaryotic cells were combined in one plasmid using polymerase chain reaction (PCR) fragments from a commercial vector, pZErO-2 (Invitrogen, Carlsbad, CA) and a eukaryotic expression vector , pJW4303 (Lu et al., 1997).

A 1853 bp fragment from pZErO2 from nt 1319 to nt 3178 included the ColE1origin of replication and the kanamycin resistance gene. A 2040 bp fragment from pJW4303 from nt 376 to nt 2416 included the CMVIE promoter with intron A, a synthetic homolog of the tissue plaminogen activator leader

(tPA), and the bovine growth hormone polyadenylation site (BGHpA). Fragments were amplified by polymerase chain reaction (PCR) with oligonucleotide primers containing SalI sites. A ligation product with the transcription cassettes for Kanamycin resistance from pZeRO2 and the eukaryotic transcription cassette form pJW4303 in opposite transcriptional orientations was identified for further development. Nucleotide numbering for this parent for the pGA vectors was started from the first bp of the 5' end of the CMV promoter .

The T0 terminator was introduced into this parent for the pGA vectors by PCR amplification of a 391 bp fragment with a BamH1 restriction endonuclease site at its 5'end and an XbaI restriction endonuclease site at its 3'end. The initial 355 bp of the fragment were sequences in the BGHpA sequence derived from the pJW4303 transcription cassette, the next 36 bases in a synthetic oligonuclotide introduced the T0 sequence and the XbaI site. The introduced T0 teminator sequences comprised the nucleotide sequence as follows:

5'-ATAAAAAACGCCCGGCGGCAACCGAGCGTTCTGAA-3' (SEQ ID NO:)

The T0 terminator containing BamH1 - XbaI fragment was substituted for the homologous fragment without the T0 terminator in the plasmid created from pXeRO 2 and pJW4303. The product was sequenced to verify the T0 orientation.

A region in the eukaryotic transcription cassette between nucleotides 1755-1845 contained the last 30bp of the reading frame for SIV nef. This region was removed from pGA by mutating the sequence at nt1858 and generating an Avr II restriction endonuclease site. A naturally occurring Avr II site is located at

nt1755. Digestion with Avr II enzyme and then religation with T4 DNA ligase allowed for removal of the SIV segment of DNA between nucleotides 1755-1845. To facilitate cloning of HIV-1 sequences, into pGA vectors a ClaI site was introduced at bp1645 and an RsrII site at bp 1743 using site directed mutagenesis. Constructions were verified by sequence analyses.

Example 2: Structure and Sequence of pGA2

pGA2, as illustrated in Fig. 3 and Fig. 4, is identical to pGA1 except for deletion of the intron A sequences from the CMVIE promoter. pGA2 was created from pGA1 by introducing a ClaI site 8 bp downstream from the mRNA cap site in the CMVIE promoter. The ClaI site was introduced using oligonucleotide-directed mutagensis using the complimentary primers

5'-CCGTCAGATCGCATCGATACGCCATCCACG-3' (SEQ ID NO:) and

5'-CGTGGATGGCGTATCGATGCGATCTGACGG-3' (SEQ ID NO:).

After insertion of the new ClaI site, pGA1 was digested with *ClaI* to remove the 946bp ClaI fragment from pGA1, and then religated to yield pGA2.

Example 3: Structure and Sequence of pGA3

pGA3 as shown in Fig. 5 and Fig. 6 is identical to pGA1 except for the introduction of a HindIII site in stead of the ClaI site at nt 1645 and a BamHI site instead of the RsrII site at nucleotide 1743.

Example 4: Comparative Expression and Immunogenicity of pGA3 and

pJW4303

To determine the efficacy of the pGA plasmids as vaccine vectors, a pGA plasmid was compared to the previously described vaccine vector pJW4303. The pJW4303 plasmid has been used for DNA vaccinations in mice, rabbits, and rhesus macaques (Robinson et al. 1999; Robinson et al., 1997; Pertmer, et al., 1995; Feltquate, et al. 1997; Torres, et al. 1999). Comparisons were done with pGA3 with a vaccine insert encoding the normal, plasma-membrane form of the A/PR/8/34 (H1N1) influenza virus hemagglutinin (pGA3/H1) and pJW4303 encoding the same fragment (pJW4303/H1). Both pGA3 and pJW4303 contain intron A upstream of influenza H1 sequences.

The pGA3/H1 and pJW4303/H1 vaccine plasmids expressed similar levels of H1 in eukaryotic cells, as summarized below:

TABLE 5: *In Vitro* Expression Levels of HA plasmids.

Plasmids	Relative HA Units	
	Supernatant	Cell Lysate
PGA3/H1	0.1±0.1	5.7±0.6
pGA vector	0.0±0.0	0.2±0.1
PJW4303/H1	0.3±0.05	4.8±0.5
pJW4303	0.0±0.0	0.1±0.1

Human embryonic kidney 293T cells were transiently transfected with 2μg of plasmid and the supernatants and cell lysates assayed for H1 using an antigen-capture ELISA. The capture antibody was a polyclonal rabbit serum against H1, and the detection antibody, polyclonal mouse sera against H1. pGA3/H1 expressed slightly more H1 than pJW4303/H1 (5.8 HA units as

opposed to 5.1 H1 units (Table 6). As expected, 90% of the H1 antigen was in the cell lysates. A comparative immunization study using pGA3/H1, and pJW4303/H1 demonstrated comparable or better immunogenicity for pGA3/H1 than pJW4303/H1 (Figure 7). Immunogenicity was assessed in BALB/c mice. In this example, mice were vaccinated with DNA coated gold particles via gene gun. Mice were primed and boosted with a low dose (0.1μg) or a high dose (1μg) of the plasmid DNAs. The booster immunization was given at 4 weeks after the priming immunization. The amount of anti-H1 IgG raised in response to immunizations was as high or higher following immunization with pGA3/H1 than following immunization with pJW4303/H1 (Figure 7). Thus the pGA vector proved to be as effective, or more effective, than the pJW4303 vector at raising immune responses.

Example 5: Immunodeficiency Virus Vaccine Inserts in pGA Vectors

Immunodeficiency virus vaccine inserts expressing virus like particles have been developed in pGA1 and pGA2. The VLP insert was designed with clade B HIV-1 sequences so that it would match HIV-1 sequences that are endemic in the United States. Within clade B, different isolates exhibit clustal diversity, with each isolate having overall similar diversity from the consensus sequence for the clade (Subbarao, Schochetman, 1996). Thus, any clade B isolate can be used as a representative sequence for other clade B isolates. HIV-1 isolates use different chemokine receptors as co-receptors. The vast majority of viruses that are undergoing transmission use the CCR-5 co-receptor (Berger, E.A., 1997). Therefore the vaccine insert was designed to have a CCR-5 using Env.

The expression of VLPs with an R5-Env by a HIV-1 DNA vaccine also has the advantage of supporting Env-mediated entry of particles into professional antigen presenting cells (APCs) such as dendritic cells and macrophages. Both dendritic cells and macrophages express the CD4 receptor and the CCR-5 co-receptor used by CCR-5-tropic (R5) HIV-1 Envs. By using an R5 Env in the vaccine, the VLP expressed in a transfected non-professional APC (for example keratinocyte or muscle cells) can gain entry into the cytoplasm of an APC by Env-mediated entry. Following entry into the cytoplasm of the APC, the VLP will be available for processing and presentation by class I histocompatibility antigens. DNA-based immunizations rely on professional APCs for antigen presentation (Corr et al., 1996; Fu, et al., 1997; Iwasaki A, et al., 1997). Much of DNA-based immunization is accomplished by direct transfection of professional APC (Condon et al., 1996; Porgador et al., 1998). Transfected muscle cells or keratinocytes serve as factories of antigen but do not directly raise immune response (Torres et al., 1997). By using an expressed antigen that is assembled and released from transfected keratinocytes or muscle cells and then actively enters professional APC, the efficiency of the immunization may be increased.

Goals in the construction of pGA2/JS2 were (i) to achieve a CCR-5-using clade B VLP with high expression, (ii) to produce a VLP that was non infectious and (iii) to minimize the size of the vaccine plasmid. Following the construction of the CCR-5-using VLP (pGA2/JS2), a derivative of JS2 was prepared that expresses an Env-defective VLP. This plasmid insert was designated JS5. Although it is anticipated that this sequence will be a less effective vaccine than

the Env-containing JS2 VLP, the non-Env containing VLP offers certain advantages for vaccination. These include the ability to monitor vaccinated populations for infection by sero-conversion to Env. Deletion of Env sequences also reduces the size of the vaccine plasmid. The DNA sequence of pGA2/JS2 is shown in Fig. 17 and that of pGA1/JS5 in Figure 18.

To achieve a VLP plasmid with high expression, candidate vaccines were constructed from 7 different HIV-1 sequences, as shown in the following table:

Table I: Comparison of candidate vaccine inserts

Plasmid designation	Sequences tested	Ability to grow plasmid	Expression of Gag	Expression of Env	Comment
BH10-VLP	BH10	good	good	good	X4 Env
6A-VLP	6A env in BH10-VLP	poor	not tested	not tested	
BAL-VLP	BAL env in BH10-VLP	good	poor	poor	
ADA-VLP	ADA env in BH10-VLP	good	good	good	chosen for vaccine, renamed pGA1/JS1
CDC-A-VLP	CDC-A env in BH10-VLP	good	good	poor	
CDC-B-VLP	CDC-B-env in BH10-VLP	good	good	good	not as favorable expression as ADA
CDC-C-VLP	CDC –C env in BH10-VLP	good	good	good	not as favorable expression as ADA

An initial construct, pBH10-VLP, was prepared from IIIb sequences that are stable in bacteria and have high expression in eukaryotic cells. The BH10 sequences were obtained from the NIH-sponsored AIDS Repository (catalog #90). The parental pBH10 was used as the template for PCR reactions to

construct pBH10-VLP.

Primers were designed to yield a Gag-Rt PCR product (5' PCR product) encompassing from 5' to 3' 105 bp of the 5' untranslated leader sequence and gag and pol sequences from the start codon for Gag to the end of the RT coding sequence. The oligonucleotide primers introduced a ClaI site at the 5' end of the PCR product and EcoRI and NheI sites at the 3' end of the PCR product. Sense primer 1 (5'-GAGCTCTATCGATGCAGGACTCGGCTTGC-3' (SEQ ID NO:)) and antisense primer 2 (5'-GGCAGGTTTTAATCGCTAGCCTATGCTCTCC-3' (SEQ ID NO:)) were used to amplify the 5'PCR product.

The PCR product for the env region of HIV-1 (3'PCR product) encompassed the vpu, tat, rev, and env sequences and the splice acceptor sites necessary for proper processing and expression of their respective mRNAs. An EcoRI site was introduced at the 5' end of this product and NheI and RsrII sites were introduced into the 3' end. Sense primer 3 (5'-GGGCAGGAGTGCTAGCC-3' (SEQ ID NO:)) and antisense primer 4 (5'-CCACACTACTTTCGGACCGCTAGCCACCC-3' (SEQ ID NO:)) were used to amplify the 3'PCR product).

The 5' PCR product was cloned into pGA1 at the ClaI and NheI sites and the identity of the construct confirmed by sequencing. The 3' PCR product was then inserted into the 5' clone at the EcoRI and NheI sites to yield pBH10-VLP. The construction of this VLP resulted in proviral sequences that lacked LTRs, integrase, vif, and vpr sequences (see Figure 8A).

Because the BH10-VLP had an X4 rather than an R5 Env, sequences encoding six different R5 Envs were substituted for env sequences in BH10-VLP. This was done by cloning EcoRI to BamHI fragments encompassing tat, rev, vpu and env coding sequences from different viral genomes into pBH10-VLP. The resulting env and rev sequences were chimeras for the substituted sequences and BH10 sequences (for example see Figure 8B). In the case of the ADA envelope, a BamHI site was introduced into the ADA sequence to facilitate substituting an EcoRI to BamHI fragment for the EcoRI to BamHI region of the BH10-VLP (Figure 8). The results of these constructions are summarized in Table 1. Of the six sequences tested, one, the 6A-VLP was found to be associated with poor plasmid growth in transformed bacteria. This plasmid was not used for further vaccine development (Table 1).

Among the plasmids exhibiting good growth in bacteria, the best expression of the VLP was found for the ADA-VLP (Table 1). In transient transfections in 293T cells, the expression of the ADA-VLP was higher than that of wt proviruses for ADA or IIIb (Fig.9). Expression was also higher than for a previous VLP-vaccine (dpol) (Richmond et al., 1998) that had successfully primed cytotoxic T-cell (Tc) responses in rhesus macaques (Kent et al., 1998).

Example 6: Safety mutations

Once the ADA-VLP had been identified as a favorable candidate for

further vaccine development, this plasmid was mutated to increase its safety for use in humans. Further mutations disabled the Zinc fingers in NC that are active in the encapsidation of viral RNA, and added point mutations to inactivate the activity of the viral reverse transcriptase and the viral protease (Fig. 8). The following table summarizes the location of the safety point mutations

Table 2. Location of safety point mutations in pGA/JS2 and pGA/JS5 to inhibit viral RNA packaging and abolish reverse transcriptase activity in vaccine constructs

GENE	REGION	FUNCTION	AMINO ACID CHANGE[1]	LOCATIO
Gag	Zn finger	Viral RNA packaging	C392S	1285/128
Gag	Zn finger	Viral RNA packaging	C392S	1294/129
Gag	Zn finger	Viral RNA packaging	C413S	1348/135
Gag	Zn finger	Viral RNA packaging	C416S	1357/135
Pol	RT	Polymerase activity	D185N	2460/246
Pol	RT	Strand transfer	W266T	2703/2704/2
Pol	RNAse H	RNAse activity	E478Q	3339

[1]Amino acid number corresponds to individual genes in HIV-1 BH10 sequence; [2]Nucleotide number in wt HIV-1 BH10 sequence

The mutations were made using a site directed mutagenesis kit (Stratagene) following the manufacturer's protocol. All mutations were confirmed by

sequencing. Primer pairs used for the mutagenesis were:

(A) C15S ZN1 5'-GGTTAAGAGCTTCAATAGCGGCAAAGAAGGGC-3' (SEQ ID NO:)

C15S ZN2 5'-GCCCTTCTTTGCCGCTATTGAAGCTCTTAACC-3' (SEQ ID NO:)

(B) C36S ZN3 5'-GGGCAGCTGGAAAAGCGGAAAGGAAGG-3' (SEQ ID NO:)

C36S ZN4 5'-CCTTCCTTTCCGCTTTTCCAGCTGCCC-3' (SEQ ID NO:)

(C) D185N RT1 5'-CCAGACATAGTTATCTATCAATACATGAACGATTTGTATGTAGG-3' (SEQ ID NO:)

D185N RT2 5'-CCTACATACAAATCGTTCATGTATTGATAGATAACTATGTCTGG-3' (SEQ ID NO:)

(D) W266T RT3 5'-GGGGAAATTGAATACCGCAAGTCAGATTTACCC-3' (SEQ ID NO:)

W266T RT4 5' GGGTAAATCTGACTTGCGGTATTCAATTTCCCC-3'

(SEQ ID NO:)

(E) E478Q RT5 5'-CCCTAACTAACACAACAAATCAGAAAACTCAGTTACAAGC-3'
(SEQ ID NO:)
E478Q RT6 5'-GCTTGTAACTGAGTTTTCTGATTTGTTGTGTTAGTTAGGG-3'
(SEQ ID NO:)

(F) D25A Prt1 5'-GGCAACTAAAGGAAGCTCTATTAGCCACAGGAGC-3'
(SEQ ID NO:)
D25Aprt2 5'-GCTCCTGTGGCTAATAGAGCTTCCTTTAGTTGCC-3'
(SEQ ID NO:)

The ADA-VLP with the zinc finger and RT mutations was found to express Gag and Env more effectively than the VLP plasmid without the mutations (Figure 10). The mutation that inactivated the protease gene markedly reduced VLP expression (Figure 10) and was not included in the further development of the vaccine plasmid. The ADA-VLP without mutations was designated JS1 and the ADA-VLP with mutations, JS2.

Example 7: Construction of the JS5 Vaccine Insert

The JS5 insert, a plasmid expressing Gag, RT, Tat, and Rev was constructed from JS2 by deleting a BglII fragment in the ADA Env (Fig. 8). This

deletion removed sequences from nt 4906-5486 of the pGA2/JS2 sequence and results in a premature stop codon in the env gene leading to 269 out of the 854 amino acids of Env being expressed while leaving the tat, rev, and vpu coding regions the RRE and splice acceptor sites intact. The DNA sequence of pGA1/JS5 is shown in Fig. 18.

Example 8: Minimizing the Size of the JS2 and JS5 Vaccine Plasmids

The JS2 and JS5 vaccine inserts were originally constructed in pGA1, a vector that contains the ~1 kb intron A of the CMVIE promoter upstream of the vaccine insert. To determine whether this intron was necessary for high levels of vaccine expression, pGA2 vectors lacking intron A were constructed expressing the JS2 and JS5 vaccine inserts. In expression tests, pGA2 proved to have as good an expression pattern as pGA1 for JS2 (Figure 11). In contrast to this result, JS5 was expressed much more effectively by pGA1 than pGA2 (Figure 11). For the JS5 insert, the absence of intron A resulted in 2-3-fold lower levels of expression than in the presence of intron A.

Example 9. Testing for the efficacy of the safety mutations in the vaccine inserts JS2 and JS5

The three point mutations in RT (Table 2) completely abolished detectable levels of reverse transcriptase activity for JS2 and JS5. A highly sensitive reverse transcriptase assay was used in which the product of reverse transcription was amplified by PCR (Yamamoto, Folks, Heneine, 1996). This assay can detect

reverse trasncriptase in as few as 10 viral particles. Reverse transcriptase assays were conducted on the culture supernatants of transiently transfected cells. Reverse transcriptase activity was readily detected for as few as 10 particles ($4x10^{-3}$ pg of p24) in the JS1 vaccine but could not be detected for the JS2 or JS5 inserts.

The deletions and zinc finger mutations in the JS2 and JS5 vaccine inserts (Table 2) reduced the levels of viral RNA in particles by at least 1000-fold. Particles pelleted from the supernatants of transiently transfected cells were tested for the efficiency of the packaging of viral RNA. The VLPs were treated with DNase, RNA was extracted and the amount of RNA standardized by p24 levels before RT PCR. The RT PCR reaction was followed by nested PCR using primers specific for viral sequences. End point dilution of the VLP RNA was compared to the signal obtained from RNA packaged in wt HIV-1 Bal virus.

Packaging for both JS2 and JS5 was restricted by the deletions in the plasmid by 500-1000-fold, as summarized below:

Table 3:Packaging of viral RNA is reduced in pGA2/JS2 and pGA1/JS5 VLPs

Vaccine Construct	Deletions/Mutations	Copies vRNA relative to wt HIV-1 b
HIV-1 bal	Wt	1
pGA1/JS1 VLP	Deleted: LTRs, int, vif, vpr, nef	.002
pGA1/JS2 VLP	Deleted: LTRs, int, vif, vpr, nef, Mutations in Zn fingers and RT	.0001
pGA1/JS4 VLP	Deleted: LTRs, int, vif, vpr, nef	.001
pGA1/JS5 VLP	Deleted: LTRs, int, vif,	.001

	vpr, nef, env; Mutations in Zn fingers and RT	

The zinc finger mutations decreased the efficiency of packaging for the JS2 particles a further 20-fold but did not further affect the efficiency of packaging for the JS5 particles. This pattern of packaging was reproducible for particles produced in independent transfections.

Example 10: Western blot analyses of protein expression.

Western blot analyses, shown in Figs. 12A-D, revealed the expected patterns of expression of pGA2/JS2 and pGA1/JS5. Both immature and mature proteins were observed in cell lysates, whereas only the mature forms of Gag and Env were found in the VLP-containing lysates (Figs. 12B and 12C). Reverse transcriptase was readily detected in cell lysates (Fig. 12D).

Example 11: pGA2/89.6 SHIV Vector Construction

Initial immunogenicity trials have been conducted with a SHIV-expressing VLP rather than the HIV-1-expressing vaccine plasmids. SHIVs are hybrids of simian and human immunodeficiency virus sequences that grow well in macaques (Li et al., 1992). By using a SHIV, vaccines that are at least partially of HIV-1 origin can be tested for efficacy in macaque models.

pGA2/89.6 (also designated as pGA2/M2) expresses sequences from SHIV-89.6 (Reimann, Li, Voss, et al., 1996; Reimann, Li, Veazey, et al., 1996). The 89.6 Env represents a patient isolate (Collman et al., 1992). The SHIV-89.6 virus is available as a highly pathogenic challenge stock, designated SHIV-89.6P (Reimann, Li, Voss, et al., 1996; Reimann, Li, Veazey, et al., 1996), which allows a rapid determination of vaccine efficacy. The SHIV-89.6P challenge can be administered via both intrarectal and intravenous routes. SHIV-89.6 and SHIV-89.6P do not generate cross-neutralizing antibody.

pGA2/89.6 (Fig.13) has many of the design features of pGA2/JS2. Both express immunodeficiency virus VLPs: HIV-1 VLP in the case of pGA2/JS2, while the VLP expressed by pGA2/89.6 is a SHIV VLP. The gag-pol sequences in pGA2/89.6 are from SIV239, while the tat, rev, and env sequences are from HIV-1-89.6. pGA2/89.6 also differs from pGA2/JS2 in that the integrase, vif and vpr sequences have not been deleted, nor has the reverse transcriptase gene been inactivated by point mutations. Finally, the zinc fingers in NC have been inactivated by a deletion and not by point mutations.

pGA1/Gag-Pol was also constructed to allow evaluation of the protective efficacy of a Gag-Pol expressing vector with the Gag-Pol-Env expresssing pGA2/89.6. This vector was constructed from pGA1/JS5 and pGA2/89.6 (Fig.13).

Example 12: Comparison of the expression of pGA2/89.6 SHIV plasmid with pGA2/JS2 expression

Both pGA2/89.6 and pGA1/Gag-Pol expressed similar levels of Gag as pGA2/JS2. Comparative studies for expression were performed on transiently transfected 293T cells. Analyses of the lysates and supernatants of transiently transfected cells revealed that both plasmids expressed similar levels of capsid antigen (Figure 14). The capsid proteins were quantified using commercial antigen capture ELISA kits for HIV-1 p24 and SIV p27.

Example 13: pGA2/89.6 SHIV vaccine protocol

A rhesus macaque model was used to investigate the ability of systemic DNA priming followed by a recombinant MVA (rMVA) booster to protect against a mucosal challenge with the SHIV-89.6P challenge strain (Amara et al, 2001).

The DNA component of the vaccine (pGA2/89.6) was made as described in Example 11 and and expressed eight immunodeficiency virus proteins (SIV Gag, Pol, Vif, Vpx, and Vpr and HIV Env, Tat, and Rev) from a single transcript using the subgenomic splicing mechanisms of immunodeficiency viruses. The rMVA booster (89.6-MVA) was provided by Dr. Bernard Moss (NIH) and expresses both the HIV 89.6 Env and the SIV 239 Gag-Pol, inserted into deletion II and deletion III of MVA respectively, under the control of vaccinia virus early/late promoters (Wyatt and Moss, unpublished results). The 89.6 Env protein was truncated for the C-terminal 115 amino acids of gp41. The modified H5 promoter controlled the expression of both foreign genes.

The vaccination trial compared i.d. and i.m. administration of the DNA vaccine and the ability of a genetic adjuvant, a plasmid expressing macaque GM-

CSF, to enhance the immune response raised by the vaccine inserts. Vaccination was accomplished by priming with DNA at 0 and 8 weeks and boosting with rMVA at 24 weeks. For co-delivery of a plasmid expressing GM-CSF, 1-100 μl i.d. inoculation was given with a solution containing 2.5 mg of pGA2/89.6 and 2.5 mg per ml of pGM-CSF.

I.d. and i.m. deliveries of DNA were compared for two doses, 2.5 mg and 250 μg of DNA. Four vaccine groups of six rhesus macaques were primed with either 2.5 mg (high-dose) or 250 μg (low-dose) of DNA by intradermal (i.d.) or intramuscular (i.m.) routes using a needleless jet injection device (Bioject, Portland OR). The 89.6-MVA booster immunization ($2x10^8$ pfu) was injected with a needle both i.d. and i.m. A control group included two mock immunized animals and two naive animals. The vaccination protocol is summarized as follows:

Table 4. Vaccination Trial

Group, (# macaque)	Prime at 0 and 8 weeks	Immunogen	Boost at 24 weeks	Immunogen
1 (6)	i.d. bioject	2.5 mg VLP DNA	i.d.+i.m.	MVA gag-pol-env
2 (6)	i.m. bioject	2.5 mg VLP DNA	i.d. + i.m.	MVA gag-pol-env
3 (6)	i.d bioject	250 ug VLP DNA	i.d. + i.m	MVA gag-pol-env
4 (6)	i.m. bioject	250 ug VLP DNA	i.d. + i.m.	MVA gag-pol-env
5 (6)	i.d. bioject	2.5 mg gag-pol DNA	i.d. + i.m.	MVA gag-pol
6 (6)	i.d. bioject	250 ug gag-pol DNA	i.d. + i.m.	MVA gag-pol
7 (6)	i.d bioject	250 ug VLP DNA+ 250 ug GM-CSF DNA	i.d. + i.m.	MVA gag-pol-env
8 (5)	i.d. bioject i.d. + i.m.control MVA	2.5 mg control DNA control MVA	i.d. + i.m.	control MVA
9 (4)	i.d., bioject	250 ug control DNA + 250 ug GM-CSF DNA	i.d. + i.m.	MVA gag-pol-env
10 (6)	i.d. + i.m.	MVA gag-pol-env	i.d.+ i.m.	MVA gag-pol-env

VLP DNA expresses all SHIV-89.6 proteins except Nef, truncated for LTRs, 2nd ZN++ finger, mutated to express cell surface Env; gag-pol DNA expresses SIV mac 239 gag-pol; MVA gag-pol-env expresses 89.6 truncated env and SIV mac 239 gag-pol; MVA gag-pol expresses SIVmac239 gag-pol; MVA dose is $1x10^8$ pfu

Animals were challenged seven months after the rMVA booster to test whether the vaccine had generated long-term immunity. Because most HIV-1 infections are transmitted across mucosal surfaces, an intrarectal challenge was administered to test whether the vaccine could control a mucosal immunodeficiency virus challenge. Briefly, the challenge stock (5.7×10^9 copies of viral RNA per ml) was produced by one i.v. followed by one intrarectal passage in rhesus macaques of the original SHIV-89.6P stock. Lymphoid cells

were harvested from the intrarectally infected animal at peak viremia, CD8-depleted and mitogen-stimulated for stock production. Prior to intrarectal challenge, fasted animals were anesthetized (ketamine, 10mg/kg) and placed on their stomach with the pelvic region slightly elevated. A feeding tube [8Fr (2.7mm) x 16 inches (41 cm), Sherwood Medical, St. Louis, MO] was inserted into the rectum for a distance of 15-20 cm. Following insertion of the feeding tube, a syringe containing 20 intrarectal infectious doses in two ml of RPMI-1640 plus 10% fetal bovine serum (FBS) was attached to the tube and the inoculum slowly injected into the rectum. Following delivery of the inoculum, the feeding tube was flushed with 3.0 ml of RPMI without fetal calf serum and then slowly withdrawn. Animals were left in place, with pelvic regions slightly elevated, for a period of ten minutes following the challenge.

Example 14: Vaccine-raised T-cell responses

DNA priming followed by rMVA boosting generated high frequencies of virus-specific T cells that peaked at one week following the rMVA booster, as shown in Fig. 15. The frequencies of T cells recognizing the Gag-CM9 epitope were assessed using Mamu-A*01- tetramers; and the frequencies of T cells recognizing epitopes throughout Gag and Env, using pools of overlapping Gag and Env peptides and an enzyme linked immunospot (ELISPOT) assay.

For tetramer analyses, approximately 1×10^6 PBMC were surface stained with antibodies to CD3 (FN-18, Biosource International, Camarillo, CA), CD8 (SK1, Becton Dickinson, San Jose, CA), and Gag-CM9 (CTPYDINQM)-Mamu-A*01 tetramer conjugated to FITC, PerCP and APC respectively, in a volume of 100 µl at 8-10°C for 30 min. Cells were washed twice with cold PBS containing

2% FBS, fixed with 1% paraformaldehyde in PBS and analyses acquired within 24 hrs. on a FACScaliber (Becton Dickinson, San Jose, CA). Cells were initially gated on lymphocyte populations using forward scatter and side scatter and then on CD3 cells. The CD3 cells were then analyzed for CD8 and tetramer-binding cells. Approximately 150,000 lymphocytes were acquired for each sample. Data were analyzed using FloJo software (Tree Star, Inc. San Carlos, CA).

For IFN-γ ELISPOTs, MULTISCREEN 96 well filtration plates (Millipore Inc. Bedford, MA) were coated overnight with anti-human IFN-γ antibody (Clone B27, Pharmingen, San Diego, CA) at a concentration of 2 μg/ml in sodium bicarbonate buffer (pH 9.6) at 8-10°C. Plates were washed two times with RPMI medium then blocked for one hour with complete medium (RPMI containing 10% FBS) at 37°C. Plates were washed five more times with plain RPMI medium and cells were seeded in duplicate in 100 μl complete medium at numbers ranging from $2x10^4$ to $5x10^5$ cells per well. Peptide pools were added to each well to a final concentration of 2 μg/ml of each peptide in a volume of 100 μl in complete medium. Cells were cultured at 37°C for about 36 hrs under 5% CO_2. Plates were washed six times with wash buffer (PBS with 0.05% Tween-20) and then incubated with 1 μg of biotinylated anti-human IFN-γ antibody per ml (clone 7-86-1, Diapharma Group Inc., West Chester, OH) diluted in wash buffer containing 2% FBS. Plates were incubated for 2 hrs at 37°C and washed six times with wash buffer. Avidin-HRP (Vector Laboratories Inc, Burlingame, CA) was added to each well and incubated for 30-60 min at 37°C. Plates were washed six times with wash buffer and spots were developed using stable DAB as substrate (Research Genetics Inc., Huntsville, AL). Spots were counted using a stereo dissecting microscope. An ovalbumin peptide (SIINFEKL) was included as a control in each analysis. Background spots for the ovalbumin peptide were

generally <5 for $5x10^5$ PBMC s. This background when normalized for $1x10^6$ PBMC is <10. Only ELISPOT counts of twice the background (≥20) were considered significant. The frequencies of ELISPOTs are approximate because different dilutions of cells have different efficiencies of spot formation in the absence of feeder cells *(34)*. The same dilution of cells was used for all animals at a given time point, but different dilutions were used to detect memory and peak effector responses.

Simple linear regression was used to estimate correlations between post-booster and post-challenge ELISPOT responses, between memory and post-challenge ELISPOT responses, and between log viral loads and ELISPOT frequencies in vaccinated groups. Comparisons between vaccine and control groups were performed by means of 2-sample t-tests using log viral load and log ELISPOT responses. Comparisons of ELISPOTs or log viral loads between *A*01* and non *A*01* macaques were done using 2-sample t-tests. Two-way analyses of variance were used to examine the effects of dose and route of administration on peak DNA/MVA ELISPOTs, memory DNA/MVA ELISPOTs, and on logarithmically transformed Gag antibody data.

Gag-CM9 tetramer analyses were restricted to macaques that expressed the *Mamu- A*01* histocompatibility type, whereas ELISPOT responses did not depend on a specific histocompatibility type. Temporal T cell assays were designed to score both the acute (peak of effector cells) and long-term (memory) phases of the T cell response (Fig 15A). As expected, the DNA immunizations raised low levels of memory cells that expanded to high frequencies within one week of the rMVA booster (Fig. 15). In *Mamu-A*01* macaques, cells specific to the Gag-CM9 epitope expanded to frequencies as high as 19% of total CD8 T cells (see animal 2 Fig 15B). This peak of specific cells underwent a >10-fold

contraction into the DNA/MVA memory pool (Figs. 15A and B). ELISPOTs for three pools of Gag peptides also underwent a major expansion (frequencies up to 4000 spots for 1×10^6 PBMC) before contracting into the DNA/MVA memory response (Fig. 15C). The frequencies of ELISPOTs were the same in macaques with and without the *A*01* histocompatibility type (P>0.2.). At both peak and memory phases of the vaccine response, the rank order for the height of the ELISPOTs in the different vaccine groups was 2.5 mg i.d > 2.5 mg i.m. > 250 μg i.d. > 250 μg i.m. (Fig.15C). The IFN-γ-ELISPOTs included both CD4 and CD8 cells (work in progress). Gag-CM9-specific CD8 cells had good lytic activity following restimulation with peptide (data not shown).

Impressively, in the outbred population of animals, pools of peptides throughout Gag and Env stimulated IFN-γ-ELISPOTs (Fig. 16A). The breadth of the cellular response was tested at 25 weeks after the rMVA boost, a time when vaccine-raised T cells were in memory. Seven out of 7 pools of Gag peptides and 16 out of 21 pools of Env peptides were recognized by T cells in vaccinated animals. Of the five Env pools that were not recognized, two have been recognized in a macaque DNA/MVA vaccine trial at the U.S. Centers for Disease Control (data not shown). The remaining three (pools 19-21) had been truncated in our immunogens (Amara et al, 2001, submitted) and served as negative controls. Gag and Env ELISPOTs had overall similar frequencies in the DNA/MVA memory response (Fig. 16B). The greatest breadth of response was in high-dose i.d. DNA-primed animals where on average 10 peptide pools (4.5 Gag and 5.3 Env) were recognized. The rank order of the vaccine groups for breadth was the same as for the peak DNA/MVA response: 2.5 mg i.d. > 2.5 mg i.m. > 250 μg i.d. > 250 μg i.m. (Fig.16B).

Example 15: Challenge and protection against AIDS

The highly pathogenic SHIV-89.6P challenge was administered intrarectally at 7 months after the rMVA booster, when vaccine-raised T cells were in memory (Fig 15).

Determination of SHIV copy number: viral RNA from 150 μl of ACD anticoagulated plasma was directly extracted with the QIAamp Viral RNA kit (Qiagen), eluted in 60 μl AVE buffer, and frozen at -80°C until SHIV RNA quantitation was performed. 5 μl of purified plasma RNA was reverse transcribed in a final 20 μl volume containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 4 mM $MgCl_2$, 1 mM each dNTP, 2.5 μM random hexamers, 20 units MultiScribe RT, and 8 units RNase inhibitor. Reactions were incubated at 25°C for 10 min., followed by incubation at 42°C for 20 min. and inactivation of reverse transcriptase at 99°C for 5 min. The reaction mix was adjusted to a final volume of 50μl containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 4 mM $MgCl_2$, 0.4 mM each dNTP, 0.2 μM forward primer, 0.2 μM reverse primer, 0.1 μM probe and 5 units AmpliTaq Gold DNA polymerase (all reagents from Perkin Elmer Applied Biosystems, Foster City, CA). The primer sequences within a conserved portion of the SIV *gag* gene are the same as those described previously (Staprans, S., et al., 1996).

A Perkin Elmer Applied Biosystems 7700 Sequence Detection System was used with the PCR profile: 95°C for 10 min., followed by 40 cycles at 93°C for 30 sec., 59.5°C for 1 min. PCR product accumulation was monitored using the 7700 sequence detector and a probe to an internal conserved *gag* gene sequence, where FAM and Tamra denote the reporter and quencher dyes. SHIV RNA copy number was determined by comparison to an external standard curve

consisting of virion-derived SIVmac239 RNA quantified by the SIV bDNA method (Bayer Diagnostics, Emeryville, CA). All specimens were extracted and amplified in duplicate, with the mean result reported. With a 0.15-ml plasma input, the assay has a sensitivity of 10^3 copies RNA/ml plasma, and a linear dynamic range of 10^3 to 10^8 RNA copies ($R^2 = 0.995$). The intra-assay coefficient of variation is <20% for samples containing >10^4 SHIV RNA copies/ml, and <25% for samples containing 10^3 - 10^4 SHIV RNA copies/ml. In order to more accurately quantitate low SHIV RNA copy number in vaccinated animals at weeks 16 and 20, the following modifications to increase the sensitivity of the SHIV RNA assay were made: 1) Virions from $\leq$1ml of plasma were concentrated by centrifugation at 23,000g, 10°C for 150 minutes and viral RNA was extracted; 2) A one-step RT-PCR method was used. Absolute SHIV RNA copy numbers were determined by comparison to the same SIVmac239 standards. These changes provided a reliable quantitation limit of 300 SHIV RNA copies/ml, and gave SHIV RNA values that were highly correlated to those obtained by the first method used ($r = 0.91$, $p<0.0001$).

Challenge results: The challenge infected all of the vaccinated and control animals. However, by two weeks post-challenge, titers of plasma viral RNA were at least 10-fold lower in the vaccine groups (geometric means of $1\text{x}10^7$ to $5\text{x}10^7$) than in the control animals (geometric mean of $4\text{x}10^8$)(Fig. 19A). By 8 weeks post-challenge, both high-dose DNA-primed groups and the low-dose i.d. DNA-primed group had reduced their geometric mean loads to about 1000 copies of viral RNA per ml. At this time the low-dose i.m. DNA-primed group had a geometric mean of $6\text{x}10^3$ copies of viral RNA and the non-vaccinated controls, a geometric mean of $2 \text{ x } 10^6$. By 20 weeks post-challenge, even the low-dose i.m. group had reduced its geometric mean copies of viral RNA to 1000. At this time,

the unvaccinated controls were succumbing to AIDS. Among the 24 vaccinated animals, only one animal, in the low dose i.m. group, had intermittent viral loads above 1×10^4 copies per ml (Fig 19D).

The rapid reduction of viral loads protected the vaccinated macaques against the loss of CD4 cells and the rapid onset of AIDS (Figs. 19B, 19C, 19E). By 5 weeks post-challenge, all of the non-vaccinated controls had undergone the profound depletion of CD4 cells that is characteristic of SHIV-89.6P infections (Fig 19B). All of the vaccinated animals maintained their CD4 cells with the exception of animal 22 (see above), which underwent a slow CD4 decline (Fig 19E). By 23 weeks post-challenge, three of the four control animals had succumbed to AIDS (Fig. 19C). These animals had variable degrees of enterocolitis with diarrhea, cryptosporidiosis, colicystitis, enteric campylobacter infection, spenomegaly, lymphadenopathy, and SIV-associated giant cell pneumonia. In contrast, all 24 vaccinated animals have maintained their health.

Intracellular cytokine assays: approximately 1×10^6 PBMC were stimulated for one hour at 37°C in 5 ml polypropylene tubes with 100 µg of Gag-CM9 peptide (CTPYDINQM) per ml in a volume of 100 µl RPMI containing 0.1% BSA and anti-human CD28 and anti-human CD49d (Pharmingen, Inc. San Diego, CA) costimulatory antibodies (1 µg/ml). 900 µl RPMI containing 10% FBS and monensin (10 µg/ml) was added and the cells cultured for an additional 5 hrs at 37°C at an angle of 5 degrees under 5% CO_2. Cells were surface stained with antibodies to CD8 conjugated to PerCP (clone SK1, Becton Dickinson) at 8°-10°C for 30 min., washed twice with cold PBS containing 2% FBS, fixed and permeabilized with Cytofix/Cytoperm solution (Pharmingen, Inc.). Cells were then incubated with antibodies to human CD3 (clone FN-18, Biosource International, Camarillo, CA) and IFN-γ (Clone B27, Pharmingen) conjugated to

FITC and PE, respectively, in Perm wash solution (Pharmingen) for 30 min at 4°C. Cells were washed twice with Perm wash once with plain PBS, resuspended in 1% para-formaldehyde in PBS. Approximately 150,000 lymphocytes were acquired on the FACScaliber and analyzed using FloJo software.

Proliferation assay: Approximately 0.2 million PBMC were stimulated with appropriate antigen in triplicate in a volume of 200 μl for five days in RPMI containing 10% FCS at 37°C under 5% CO_2. Supernatants from 293T cells transfected with the DNA expressing either SHIV-89.6 Gag and Pol or SHIV-89.6 Gag, Pol and Env were used directly as antigens. Supernatants from mock DNA (vector alone) transfected cells served as negative controls. On day six cells were pulsed with 1 μCi of tritiated-thymidine per well for 16-20 hrs. Cells were harvested using an automated cell harvester (TOMTEC, Harvester 96, Model 1010, Hamden, CT) and counted using a Wallac 1450 MICROBETA Scintillation counter (Gaithersburg, MD). Stimulation indices are the counts of tritiated-thymidine incorporated in PBMC stimulated with 89.6 antigens divided by the counts of tritiated-thymidine incorporated by the same PBMC stimulated with mock antigen.

Post-challenge T cell results: Containment of the viral challenge was associated with a burst of antiviral T cells (Fig.15; Fig.20A). At one-week post challenge, the frequency of tetramer+ cells in the peripheral blood had decreased, potentially reflecting the recruitment of specific T cells to the site of infection (Fig. 20A). However, by two weeks post-challenge, tetramer+ cells in the peripheral blood had expanded rapidly, to frequencies as high, or higher, than after the MVA booster (Figs. 15, 20A). The majority of the tetramer+ cells produced IFN-γ in response to a 6-hour stimulation with peptide Gag-CM9 (Fig. 20B) and did not have the "stunned" IFN-γ negative phenotype sometimes

observed in chronic viral infections. The post-challenge burst of T cells contracted concomitant with the decline of the viral load. By 12 weeks post-challenge, virus-specific T cells were present at approximately one tenth of their peak height (Figs. 15A, 20A, and data not shown). The height of the peak DNA/MVA-induced ELISPOTs presaged the height of the post-challenge T cell response as measured by ELISPOTs ($r = +0.79$, $P<0.0001$). In contrast to the vigorous secondary response in the vaccinated animals, the naive animals mounted a modest primary response (Figs. 15B, 15C and 20A). Tetramer+ cells peaked at less than 1% of total CD8 cells (Fig. 20A), and IFN-γ-producing T cells were present at a mean frequency of about 300 as opposed to the much higher frequencies of 1000 to 6000 in the vaccine groups (Fig. 15C) ($P<0.05$). The tetramer+ cells in the control group, like those in the vaccine group, were largely IFN-γ producing following stimulation with the Gag-CM9 peptide (Fig. 20B). By 12 weeks post challenge, 3 of the 4 controls had undetectable levels of IFN-γ-producing T cells (data not shown). This rapid loss of anti-viral CD8 cells in the presence of high viral loads may reflect the lack of CD4 help.

T cell proliferative responses demonstrated that virus-specific CD4 cells had survived the challenge and were available to support the antiviral immune response (Fig. 20C). At 12 weeks post-challenge, mean stimulation indices for Gag-Pol-Env or Gag-Pol proteins ranged from 35 to 14 in the vaccine groups but were undetectable in the control group. Consistent with the proliferation assays, intracellular cytokine assays demonstrated the presence of virus-specific CD4 cells in vaccinated but not control animals (data not shown). The overall rank order of the vaccine groups for the magnitude of the proliferative response was 2.5 mg i.d. > 2.5 mg i.m. > 250 μg i.d. > 250 μg i.m.

Preservation of lymph nodes: At 12 weeks post-challenge, lymph nodes

from the vaccinated animals were morphologically intact and responding to the infection whereas those from the infected controls had been functionally destroyed (Fig. 5). Nodes from vaccinated animals contained large numbers of reactive secondary follicles with expanded germinal centers and discrete dark and light zones (Fig. 5A). By contrast, lymph nodes from the non-vaccinated control animals showed follicular and paracortical depletion (Fig. 5B), while those from unvaccinated and unchallenged animals displayed normal numbers of minimally reactive germinal centers (Fig. 5C). Germinal centers occupied < 0.05% of total lymph node area in the infected controls, 2% of the lymph node area in the uninfected controls, and up to 18 % of the lymph node area in the vaccinated groups (Fig. 5D). The lymph node area occupied by germinal centers was about two times greater for animals receiving low-dose DNA priming than for those receiving high-dose DNA priming, suggesting more vigorous immune reactivity in the low-dose animals (Fig. 5D). At 12 weeks post-challenge, *in situ* hybridization for viral RNA revealed rare virus-expressing cells in lymph nodes from 3 of the 24 vaccinated macaques, whereas virus-expressing cells were readily detected in lymph nodes from each of the infected control animals (Fig. 5E). In the controls, which had undergone a profound depletion in CD4 T cells, the cytomorphology of infected lymph node cells was consistent with a macrophage phenotype (data not shown).

Temporal antibody response: ELISAs for total anti-Gag antibody used bacterial produced SIV gag p27 to coat wells (2 μg per ml in bicarbonate buffer). ELISAs for anti-Env antibody used 89.6 Env produced in transiently transfected 293T cells captured with sheep antibody against Env (catalog number 6205; International Enzymes, Fairbrook CA). Standard curves for Gag and Env ELISAs were produced using serum from a SHIV-89.6-infected macaque with known

amounts of anti-Gag or anti-Env IgG. Bound antibody was detected using goat anti-macaque IgG-PO (catalog # YNGMOIGGFCP, Accurate Chemical, Westbury, NY) and TMB substrate (Catalog # T3405, Sigma, St. Louis, MO). Sera were assayed at 3-fold dilutions in duplicate wells. Dilutions of test sera were performed in whey buffer (4% whey and 0.1% tween 20 in 1X PBS). Blocking buffer consisted of whey buffer plus 0.5% non-fat dry milk. Reactions were stopped with 2M H_2SO_4 and the optical density read at 450 nm. Standard curves were fitted and sample concentrations were interpolated as μg of antibody per ml of serum using SOFTmax 2.3 software (Molecular Devices, Sunnyvale, CA).

Results showed that the prime/boost strategy raised low levels of anti-Gag antibody and undetectable levels of anti-Env antibody (Fig. 22). However, post-challenge, antibodies to both Env and Gag underwent anamnestic responses with total Gag antibody reaching heights approaching one mg per ml and total Env antibody reaching heights of up to 100 μg per ml (Figs. 22A and B).

By two weeks post-challenge, neutralizing antibodies for the 89.6 immunogen, but not the SHIV-89.6P challenge were present in the high-dose DNA-primed groups (geometric mean titers of 352 in the i.d. and 303 in the i.m. groups) (Fig. 22C). By 5 weeks post-challenge, neutralizing antibody to 89.6P had been generated (geometric mean titers of 200 in the high-dose i.d. and 126 in the high-dose i.m. group) (Fig. 22D) and neutralizing antibody to 89.6 had started to decline. Thus, priming of an antibody response to 89.6 did not prevent a B cell response leading to neutralizing antibody for SHIV-89.6P. By 16 to 20 weeks post-challenge, antibodies to Gag and Env had fallen in most animals (Figs. 22A and B). This would be consistent with the control of the virus infection.

T cells correlate with protection. The levels of plasma viral RNA at both

two and three weeks post-challenge correlated inversely with the peak pre-challenge frequencies of DNA/MVA-raised IFN-γ ELISPOTs ($r=-0.53$, $P=0.008$ and $r=-0.70$, $P=0.0002$ respectively) (Fig. 23A).

Importantly, these correlations were observed during the time the immune response was actively reducing the levels of viremia. At later times post-challenge, the clustering of viral loads at or below the level of detection precluded correlations. Correlations also were sought between viral load and post-challenge ELISPOT, proliferative, and neutralizing antibody responses. The levels of IFN-γ ELISPOTS at two weeks post-challenge correlated with the viral load at 3 weeks post-challenge ($r=-0.51$, $P=0.009$) (data not shown). Post-challenge proliferative and neutralizing antibody responses did not correlate with viral loads.

Dose and route: The dose of DNA had significant effects on both cellular and humoral responses ($P<0.05$) while the route of DNA administration had a significant effect only on humoral responses (Figs. 23 C-E). The intradermal route of DNA delivery was about 10 times more effective than the intramuscular route for generating antibody to Gag ($P=0.02$) (Fig. 23E). Within our data set, i.d. DNA injections were about 3 times more effective at priming the height and breadth of virus-specific T cells (Figs. 23C and D). However, these differences were not significant (height, $P=0.2$; breadth, $P=0.08$). Interestingly, the route and dose of DNA had no significant effect on the level of protection. At 20 weeks post-challenge, the high-dose DNA-primed animals had slightly lower geometric mean levels of viral RNA ($7x10^2$ and $5x10^2$) than the low-dose DNA-primed animals ($9x10^2$ and $1x10^3$). The animal with the highest intermittent viral loads (macaque 22) was in the low dose i.m.-primed group (Fig. 19D). Thus, the low dose i.m.-primed group, which was slow to control viremia (Fig. 19A), may have poorer long term protection. The breadth of the response did not have an

immediate effect on the containment of viral loads, but with time may affect the frequency of viral escape.

These results clearly demonstrate that a multiprotein DNA/MVA vaccine can raise a memory immune response capable of controlling a highly virulent mucosal immunodeficiency virus challenge. Our excellent levels of viral control are more favorable than have been achieved using only DNA or rMVA vaccines (Egan et al., 2000; I. Ourmanov et al., 2000) and comparable to those obtained for DNA immunizations adjuvanted with interleukin-2 (Barouch et al., 2000). All of these previous studies have used more than three vaccine inoculations, none have used mucosal challenges, and most have challenged at peak effector responses and not allowed a prolonged post vaccination period to test for "long term" efficacy as was done in our study. Our results also demonstrate for the first time that vaccine-raised T cells, as measured by IFN-γ ELISPOTs, are a correlate for the control of viremia. This relatively simple assay can now be used for preclinical evaluation of DNA and MVA immunogens for HIV-1, and should be able to be used as a marker for the efficacy of clinical trials in humans.

The DNA/MVA vaccine did not prevent infection. Rather, the vaccine controlled the infection, rapidly reducing viral loads to near or below 1000 copies of viral RNA per ml of blood. Containment, rather than prevention of infection, affords the virus the opportunity to establish a chronic infection (Chun et al., 1998). Nevertheless, by rapidly reducing viral loads, a multiprotein DNA/MVA vaccine will extend the prospect for long-term non-progression and limit HIV transmission.

Example 16: Gag-Pol Vaccine Trial

A trial using Gag-Pol rather than Gag-Pol-Env expressing immunogens

was conducted to determine the importance of including Env in the vaccine (see Fig 27 for constructs). A vaccine that did not include Env would have certain advantages in the field, such as the ability to screen for anti-Env antibody as a marker for infection. This trial used pGA1/Gag-Pol and a rMVA expressing the Gag-Pol sequences of SIV239 (MVA/Gag-Pol) supplied by Dr. Bernard Moss (NIH-NIAID)

The "Gag-Pol" immunogens were administered using the schedule described in Example

13 above for the "Gag-Pol-Env" (pGA2/89.6 MVA/89.6) immunogens (see Table 4, Groups 5 and 6). The same doses of DNA, 2.5 mg and 250 μg, were used to prime a high dose and a low dose group and administration was via an intradermal route. As in the previous vaccine trial described in examples 13 – 15, two to three mamu A*01 macaques were included in each trial group. T cell responses were followed for those specific for the p11c-m epitope using the p11c-m tetramers and using ELISPOTs stimulated by pools of overlapping peptides, as described in the above Examples.

Following immunization, vaccine recipients showed anti-Gag T cell responses similar to those observed in the Gag-Pol-Env vaccine trial. Animals were challenged intrarectally with SHIV-89.6P at 7.5 months following the rMVA booster (Fig. 28). In contrast to the Gag-Pol-Env vaccine protocol, which protected animals against the rapid loss of CD4 cells, the Gag-Pol animals uniformly lost CD4 cells (Figs 28B and 28D). This loss was most pronounced in the group receiving the low dose i.d. DNA prime. Consistent with the loss of CD4

cells, the Gag-Pol DNA-immunized groups were also less effective at reducing their viral loads than the Gag-Pol-Env groups (Figs.28A and 28C). Geometric mean viral loads for these groups were 10-100-fold higher at 3 weeks post challenge and 10 fold higher at 5 weeks post challenge. These results demonstrate that the Env gene plays an important role in protecting CD4 cells and reducing the levels of viral RNA in challenged animals. The results also show that Gag-Pol-Env DNA/MVA vaccines function more effectively than Gag-Pol DNA/MVA vaccines in protecting recipients against a virulent challenge.

Example 17: Measles inserts

A DNA vaccine expressing a fusion of measles H and the C3d component of complement was used to determine if vaccination could achieve earlier and more efficient anti-H antibody responses. In prior studies in mice by Dempsey *et al.*, the fusion of two or three copies of C3d to a model antigen, hen egg lysozyme increased the efficiency of immunizations by more than 1000-fold (Dempsey et al, 1996). This resulted in more rapid appearance of hemagglutination inhibition (HI) activity and protective immunity (Ross et al, 2000 and Ross et al., 2001).

In the human immune system, one consequence of complement activation is the covalent attachment of the C3d fragment of the third complement protein to the activating protein. C3d in turn binds to CD21 on B lymphocytes, a molecule with B cell stimulatory functions that amplify B lymphocyte activation. In a measles H-C3d fusion protein, the H moiety of the fusion would bind to anti-H Ig receptors on B cells and signal through the B cell receptor, while the C3d moiety

of the fusion would bind to CD21 and signal through CD19. In this hypothesis, a B cell responding to an H-C3d fusion protein would undergo more effective signaling than a B cell responding to H alone. Mice vaccinated with DNA expressing a secreted H-fused to three copies of C3d (sH-3C3d) generated a more rapid appearance and higher levels of neutralizing antibody activity than DNA expressing sH only.

Plasmid DNA: pTR600, a eukaryotic expression vector, was constructed to contain two copies of the cytomegalovirus immediate-early promoter (CMV-IE) plus intron A (IA) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal (BGH poly A) for termination of transcription. The vector contains a multi-cloning site for the easy insertion of gene segments and the Col E1 origin of replication for prokaryotic replication and the Kanamycin resistance gene (Kan^r) for selection in antibiotic media (Fig. 29A).

Hemagglutinin (H) cDNA sequences from the Edmonton strain and C3d sequences were cloned as previously described and transferred into the pTR600 vaccine vector using unique restriction endonuclease sites (Fig. 29B). The secreted version was generated by deleting the transmembrane and cytoplasmic domains of H. This was accomplished using PCR to clone a fragment of the H gene in frame with an N-terminal synthetic mimic of the tissue plasminogen activator (tpA) leader sequence (Torres, et al, 2000).

The vectors expressing sH-C3d fusion proteins were generated by cloning three tandem repeats of the mouse homologue of C3d in frame at the 3' end of the sH gene as previously described (Dempsey, 1996; Ross et al, 2000; and Ross et

al, 2001). The construct design was based upon Dempsey *et al.* and used sequences from pSLG-C3d. Linkers composed of two repeats of 4 glycines and a serine {$(G_4S)_2$} were fused at the junctures of H and C3d and between each C3d repeat. Potential proteolytic cleavage sites between the junctions of C3d and the junction of sH and C3d were mutated by using *Bam HI* and *Bgl II* fusion to mutate an Arg codon to a Gly codon.

The plasmids were amplified in *Escherichia coli* strain, DH5α, purified using anion-exchange resin columns (Qiagen, Valencia, CA) and stored at –20°C in dH_20. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at 260nm and 280nm.

Mice and DNA immunizations: Six to 8 week old BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were used for inoculations. Briefly, mice were anesthetized with 0.03-0.04 ml of a mixture of 5ml ketamine HCl (100mg/ml) and 1ml xylazine (20mg/ml). Mice were immunized with two gene gun doses containing 0.5 μg of DNA per 0.5mg of approximately 1-μm gold beads (DeGussa-Huls Corp., Ridgefield Park, NJ) at a helium pressure setting of 400 psi.

Transfections and expression analysis: The human embryonic kidney cell line 293T (5 X 10^5 cells/transfection) was transfected with 2 μg of DNA using 12% lipofectamine according to the manufacture's guidelines (Life Technologies, Grand Island, NY). Supernatants were collected and stored at –20°C. Quantitative antigen capture ELISAs for H were conducted as previously

described (Cardoso et al, 1998).

For western hybridization analysis, 15 μl of supernatant or cell lysate was diluted 1:2 in SDS sample buffer (Bio-Rad, Hercules, CA) and loaded onto a 10% polyacrylamide/SDS gel. The resolved proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) and incubated with a 1:1000 dilution of polyclonal rabbit anti-HA antisera in PBS containing 0.1% Tween 20 and 1% nonfat dry milk. After extensive washing, bound rabbit antibodies were detected using a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antiserum and enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Antibody assays: A quantitative ELISA was performed to assess anti-H specific IgG levels. Briefly, Ltk^- cells constitutively expressing the H protein of MV (24) were grown in 96-well plates. Antisera dilutions were incubated with the intact cells expressing H antigen. The anti-H antibodies were allowed to bind to the cells for 30 min following which the cells were fixed in acetone (80%). The specific antibody responses were detected with biotinylated anti-mouse IgG antibodies and the streptavidine-phosphatase alkaline system (Sigma). Antibody binding to Ltk^- cells not expressing H antigen was used to standardize the system. The results were expressed as the endpoint dilution titer.

Neutralization assays. Neutralization assays were conducted on Vero cells grown in six well plates (25). Briefly, 100-200 p.f.u. of the Edmonton strain of measles virus were mixed with serial dilution of sera, incubated for 1 h at 37°C and then inoculated onto plates. Plates were incubated at 37°C for 48 h and

containing either the sH or sH-3C3d compared to transmembrane-associated forms of the antigen. Human 293T cells were transiently transfected with 2 μg of plasmid and both supernatants and cell lysates were assayed for H using an antigen capture ELISA. Approximately 75% of the H protein was secreted into the supernatant for both sH-DNA and sH-3C3d-DNA transfected cells. As expected, ~99% of the H antigen was detected in the cell lysate of cells transfected with plasmids expressing transmembrane form of H .

Antibody Response to Measles H DNA Immunizations: The sH-3C3d expressing DNA plasmids raised higher titers of ELISA antibody than sH DNA. BALB/c mice were vaccinated by DNA coated gold particles via gene gun with either a 0.1 μg or a 1 ug inoculum. At 4 and 26 weeks post vaccination, mice were boosted with the same dose of DNA given in the first immunization. The temporal pattern for the appearance of anti-H antibody showed a faster onset in mice vaccinated with the C3d fusion expressing DNA compared to mice vaccinated with sH DNA. Good titers of antibody were raised by the first immunization . These were boosted by the 2nd and 3rd immunizations. following the third immunization, titers were 5-6 times higher in the sH-3C3D vaccinated mice than in those vaccinated with sH DNA.

Neutralization assays: Examination of the serum for MV neutralization showed titers up to 1700 after the second inoculation of 0.1 μg of sH-3C3d expressing DNA. Neutralizing antibody studies performed on Vero cells detected higher titers of neutralizing activity against the prototype MV Edmonton strain in mouse sera elicited by the sH-3C3d constructs than in the sera of mice vaccinated with

plaques were counted. Neutralization titers are defined as the reciprocal dilution of sera required to reduce plaque formation by 50% or 90%. Preimmune sera served as negative controls.

Results: Two hemagglutinin-expressing vaccine plasmids were constructed in the pTR600 vector to express either a secreted form of H (sH) from the Edmonston strain or a C3d-fusion of the secreted form of H (sH-3C3d) (Fig. 29). The sH represented the entire ectodomain of H, but excluded the transmembrane and cytoplasmic region. The cloning placed the N-terminal synthetic mimic of the tissue plasminongen activator (tPA) leader sequence in frame with the H sequence. The tPA leader and H sequences were fused immediately 3' to the transmembrane domain of H. The sH-3C3d fusion protein was generated by cloning three tandem repeats of the mouse homologue of C3d in frame with the secreted H gene (Fig. 29B). The proteolytic cleavage sites, found at the junction between each C3d molecule as well as the junction between the H protein and the first C3d coding region, were destroyed by mutagenesis.

Western blot analyses revealed sH and sH-3C3d proteins of the expected sizes. Using a rabbit polyclonal antibody to MV H antisera, western blot analysis showed a broad band of ~70kD corresponding to the secreted form of H in the supernatant of transfected cells. A higher molecular weight band at ~190 kD is consistent with the projected size of the sH-3C3d fusion protein (Fig. 30). No evidence for the proteolytic cleavage of the sH-C3d fusion protein was seen by western analysis.

Measles virus H was expressed at slightly lower levels by plasmids

sH expressing DNA. Mice vaccinated with sH-3C3d expressing plasmids had a sharp rise in neutralizing antibody levels that reached a plateau by week 14. In contrast, it took a third vaccination with sH expressing DNA to elicit detectable levels of neutralizing antibodies. After 28 weeks post-vaccination, sera from mice vaccinated with sH-3C3d-DNA had neutralizing titers (>250) that could reduce plaque formation of MV infection by 90%.

The increase in height of the antibody response to H was 7-15 fold higher in mice vaccinated with the C3d protein expressing constructs compared to mice vaccinated with DNA expressing sH only. The increase in antibody response with DNA expressing sH-3C3d is even more intriguing, since this plasmid expressed ~60% as much protein as plasmid expressing sH only.

In addition to the increase in the overall antibody level, there was a faster onset of antibodies that could specifically neutralize MV in an *in vitro* infection assay. After the second immunization, detectable levels of neutralizing antibodies were observed in mice vaccinated with DNA expressing sH-3C3d. The titer of the neutralizing antibody peaked at week 14 (1700 for 50% plaque reduction), which are substantially above the minimum correlate for protection (>120 for 50% plaque reduction). In contrast, mice vaccinated with sH expressing DNA had low levels of neutralizing antibody even after the third vaccination (180 for 50% plaque reduction) (Fig. 31).

Example 18: Influenza inserts with and without -C3d

Plasmid vector construction and purification procedures have been

previously described for JW4303 (Torres, et al. 1999; Pertmer et al. 1995; Feltquate et al. 1997). In brief, influenza hemagglutinin (HA) sequences from A/PR/8/34 (H1N1) were cloned into either the pJW4303 or pGA eukaryotic expression vector using unique restriction sites.

Two versions of HA, a secreted(s) and a transmembrane (tm) associated, have been previously described (Torres et al. 1999; Feltquate et al.,1997). Vectors expressing sHA or tmHA in pJW4303 were designated pJW/sHA and pJW/tmHA respectively and the vectors expressing sHA, tmHA, or sHA-3C3d in pGA were designated pGA/sHA, pGA/tmHA, and pGA/sHA-3C3d respectively.

Vectors expressing HA-C3d fusion proteins were generated by cloning three tandem repeats of the mouse homolog of C3d and placing the three tandem repeats in-frame with the secreted HA gene. The construct designed was based upon Dempsey *et al.* (1996). Linkers composed of two repeats of 4 glycines and a serine [$(G_4S)_2$] were fused at the joints of each C3d repeat. The pGA/sHA-3C3d plasmid expressed approximately 50% of the protein expressed by the pGA/sHA vector. However, the ratio of sHA-3C3d found in the supernatant vs. the cell lysate was similar to the ratio of antigen expressed by pGA/sHA. More than 80% of the protein was secreted into the supernatant. In western analysis, a higher molecular weight band was detected at 120kDa and represented the sHA-3C3d fusion protein. Therefore, the sHA-3C3d fusion protein is secreted into the supernatant as efficiently as the sHA antigen.

Mice and DNA immunizations. Six to 8 week old BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were used for inoculations. Mice, housed in

microisolator units and allowed free access to food and water, were cared for under USDA guidelines for laboratory animals. Mice were anesthetized with 0.03-0.04 ml of a mixture of 5ml ketamine HCl (100mg/ml) and 1ml xylazine (20mg/ml). Gene gun immunizations were performed on shaved abdominal skin using the hand held Accell gene delivery system and immunized with two gene gun doses containing 0.5 μg of DNA per 0.5mg of approximately 1-μm gold beads (DeGussa-Huls Corp., Ridgefield Park, NJ) at a helium pressure setting of 400 psi.

Influenza virus challenge. Challenge with live, mouse-adapted, influenza virus (A/PR/8/34) was performed by intranasal instillation of 50 μl allantoic fluid, diluted in PBS to contain 3 lethal doses of virus, into the nares of ketamine-anesthetized mice. This method leads to rapid lung infections and is lethal to 100% of non-immunized mice. Individual mice were challenge at either 8 or 14 weeks after vaccination and monitored for both weight loss and survival. Data were plotted as the average individual weight in a group, as a percentage of pre-challenge weight, versus days after challenge.

Antibody response to the HA DNA Immunization protocol: The tmHA and sHA-3C3d expressing DNA plasmids raised higher titers of ELISA antibody than the sHA DNA. BALB/c mice were vaccinated by DNA coated gold particles via gene gun with either a 0.1 μg or 1 μg dose inoculum. At 4 weeks post vaccination, half of the mice in each group were boosted with the same dose of DNA given in the first immunization. Total anti-HA IgG induced by the sHA-3C3d- and tmHA- expressing plasmids were similar in the different experimental

mouse groups and 3-5 times higher then the amount raised by the sHA expressing plasmids (Fig. 24). In addition, the amount of anti-HA antibody elicited increased relative to the amount of DNA used for vaccination in a dose dependent manner (Fig 24E-24F). Overall, the dose response curves and temporal pattern for the appearance of anti-HA antibody were similar in the mice vaccinated with tmHA-DNA or sHA-3C3d-DNA, but lower and slower, in the mice vaccinated with sHA-DNA. As expected, the booster immunization both accelerated and increased the titers of antibodies to HA.

Avidity of mouse HA antiserum. Sodium thiocyanate (NaSCN) displacement ELISAs demonstrated that the avidity of the HA-specific antibody generated with sHA-3C3d expressing DNA was consistently higher than antibodies from sHA-DNA or tmHA-DNA vaccinated mice (Fig. 25). The avidity of specific antibodies to HA was compared by using graded concentrations NaSCN, a chaotropic agent, to disrupt antigen-antibody interactions. The binding of antibodies with less avidity to the antigen is disrupted at lower concentrations of NaSCN than that of antibodies with greater avidity to the antigen. The effective concentration of NaSCN required to release 50% of antiserum (ED_{50}) collected at 8 weeks after vaccination from sHA-DNA or tmHA-DNA boosted mice (0.1 μg dose or 1 μg dose) was ~1.20 M (Fig. 25A). In contrast, antiserum from mice vaccinated and boosted with sHA-3C3d-DNA had an ED_{50} of ~1.75 M (Fig 25B). At the time of challenge (14 weeks after vaccination), the ED_{50} had increased to ~1.8 M for antibodies from both sHA-DNA and tmHA-DNA vaccinated mice (Fig. 25C). Antibodies from mice

vaccinated with sHA-3C3d-DNA had increased to an ED_{50} of ~2.0 M (Fig 25D). These results suggest that the antibody from sHA-3C3d-DNA vaccinated mice had undergone more rapid affinity maturation than antibody from either sHA-DNA or tmHA-DNA vaccinated mice. The difference between the temporal avidity maturation of antibody for sHA-3C3d and tmHA was independent of the level of the raised antibody. Both of these plasmids had similar temporal patterns for the appearance of antibody and dose response curves for the ability to raise antibody (Fig. 25).

Hemagglutinin-Inhibition (HI) titers. Hemagglutination-inhibition assays (HI) were performed to evaluate the ability of the raised antibody to block binding of A/PR/8/34 (H1N1) to sialic acid. The HI titers were measured from serum samples harvested from mice at 8 and 14 weeks after vaccination. All boosted mice had measurable HI titers at week 14 regardless of the dose or vaccine given . The highest titers (up to 1:1200) were recorded for the sHA-3C3d-DNA vaccinated mice. Nonboosted mice showed more variation in HI titers. Nonboosted mice vaccinated with a 0.1 μg dose of either sHA-DNA or tmHA-DNA expressing plasmids had low HI titers of 1:10. In contrast, mice vaccinated with sHA-3C3d-DNA had titers greater than 1:640. The only vaccinated mice that had a measurable HI titer (1:160) at week 8 were boosted mice vaccinated with 1 μg dose sHA-3C3d-DNA. These results indicate that C3d, when fused to sHA, is able to stimulate specific B cells to increase the avidity maturation of antibody and thus the production of neutralizing antibodies to HA.

Protective efficacy to influenza challenge. Consistent with eliciting the highest

titers of HI antibody, the sHA-3C3d DNA raised more effective protection than the sHA or tmHA DNAs. To test the protective efficacy of the various HA-DNA vaccines, mice were challenged with a lethal dose of A/PR/8/34 influenza virus (H1N1) and monitored daily for morbidity (as measured by weight loss) and mortality. Weight loss for each animal was plotted as a percentage of the average pre-challenge weight versus days after challenge (Fig. 26). Virus-challenged naive mice and pGA vector only vaccinated mice showed rapid weight loss with all the mice losing >20% of their body weight by 8 days post-challenge (Fig. 26). In contrast, PBS mock-challenged mice showed no weight loss over the 14 days of observation. All boosted mice survived challenge, 14 weeks after vaccination, regardless of the dose of DNA plasmid administered. However, boosted mice vaccinated with a 0.1 μg dose of sHA-DNA did drop to 92% of their initial body weight at 8 days post-challenge before recovering (Fig. 26). In contrast, when 1 μg dose, boosted mice were challenged at 8 weeks after vaccination, the only mice to survive challenge were sHA-3C3d- and tmHA-DNA vaccinated mice, albeit with greater weight loss than was observed from mice challenged at 14 weeks after vaccination. The only 0.1 μg dose, boosted mice to survive challenge at 8 weeks after vaccination were the sHA-3C3d vaccinated mice (Fig. 26).

Among the nonboosted, 0.1 μg dose immunizations, only the sHA-3C3d-DNA vaccinated mice survived challenge at 14 weeks after vaccination (Fig. 26). All mice administered a single DNA vaccination lost weight. However, of these, the sHA-3C3d-DNA vaccinated mice lost the least weight and these mice were

the only mice to survive the lethal challenge (Fig. 26). These results demonstrate the that 3C3d protein, when fused to HA, increased the efficiency of a DNA vaccine, allowing for the reduction in dose of DNA and the number of vaccinations needed to afford protection to a lethal influenza virus challenge.

Example 19: HIV gp120 -C3d Fusion Constructs

In this study, a similar approach to that described in Example 18 was used to fuse three copies of murine C3d to the carboxyl terminus of HIV Env gp120 subunit. Using DNA vaccination, BALB/c mice were inoculated and assayed for enhanced immune responses. The fusion constructs induced higher antibody responses to Env and a faster onset of avidity maturation than did the respective wild-type gp120 sequences. These results suggest that the efficacy of DNA vaccines for raising antibody can be significantly improved by fusing proteins with C3d.

Plasmid DNA: pGA was constructed as described in Example 1 above to contain the cytomegalovirus immediate-early promoter (CMV-IE) plus intron A (IA) for initiating transcription of eukaryotic inserts and the bovine growth hormone polyadenylation signal (BGH poly A) for termination of transcription.

HIV envelope sequences from the isolates ADA, IIIB, and 89.6, encoding almost the entire gp120 region, and C3d sequences were cloned into the pGA vaccine vector using unique restriction endonuclease sites. The gp120 segment encoded a region from amino acid 32 to 465 and ended with the amino acid sequence VAPTRA. The first 32 amino acids were deleted from the N-terminus of each sgp120 and replaced with a leader sequenced from the tissue plasminogen activator (tpA). The vectors expressing sgp120-C3d fusion proteins were generated by cloning three tandem repeats of the mouse homologue of C3d in frame with the sgp120 expressing DNA. The construct design was based upon Dempsey *et al* (1996). Linkers composed of two repeats of 4 glycines and a serine $\{(G_4S)_2\}$ were fused at the junctures of HA and C3d and between each C3d repeat. Potential proteolytic cleavage sites between the junctions of C3d and the junction of 3C3d were mutated by ligating *Bam HI* and *Bgl II* restriction endonuclease sites to mutate an Arg codon to a Gly codon.

The plasmids were amplified in *Escherichia coli* strain-DH5α, purified using anion-exchange resin columns (Qiagen, Valencia, CA) and stored at –20°C in dH_20. Plasmids were verified by appropriate restriction enzyme digestion and gel electrophoresis. Purity of DNA preparations was determined by optical density reading at 260nm and 280nm.

Mice and DNA immunizations: Six to 8 week old BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) were vaccinated as described in Example 17 above. Briefly, mice were immunized with two gene gun doses containing 0.5 µg of DNA per 0.5mg of approximately 1-µm gold beads (DeGussa-Huls Corp.,

Ridgefield Park, NJ) at a helium pressure setting of 400 psi.

Transfections and expression analysis and western hybridization experiments were conducted as described in Example 17, except that the nitrocellulose membranes were incubated with a 1:1000 dilution of polyclonal human HIV-infected patient antisera in PBS containing 0.1% Tween 20 and 1% nonfat dry milk. After extensive washing, bound human antibodies were detected using a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-human antiserum and enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

ELISA and avidity assays: An endpoint ELISA was performed to assess the titers of anti-Env IgG in immune serum using purified HIV-1-IIIB gp120 CHO-expressed protein (Intracell) to coat plates as described (Richmond et al., 1998). Alternatively, plates were coated with sheep anti-Env antibody (International Enzymes Inc., Fallbrook, CA) and used to capture sgp120 produced in 293T cells that were transiently transfected with sgp120 expression vectors. Mouse sera from vaccinated mice was allowed to bind and subsequently detected by anti-mouse IgG conjugated to horseradish peroxidase. Endpoint titers were considered positive that were two fold higher than background. Avidity ELISAs were performed similarly to serum antibody determination ELISAs up to the addition of samples and standards. Samples were diluted to give similar concentrations of specific IgG by O.D. Plates were washed three times with 0.05% PBS-Tween 20. Different concentrations of the chaotropic agent, sodium thiocyanate (NaSCN) in PBS, were then added (0M, 1 M, 1.5 M, 2 M, 2.5 M, and 3 M NaSCN). Plates were allowed to stand at room temperature for 15 minutes and then washed six times with PBS-Tween 20. Subsequent steps were performed similarly to the serum antibody determination ELISA and percent of initial IgG calculated as a percent of the initial O.D. All assays were done in triplicate.

Neutralizing antibody assays: Antibody-mediated neutralization of HIV-1 IIIB and 89.6 was measured in an MT-2 cell-killing assay as described previously (Montefiori et al., 1988). Briefly, cell-free virus (50 µl containing 10^8 $TCID_{50}$ of virus) was added to multiple dilutions of serum samples in 100 µl of growth medium in triplicate wells of 96-well microtiter plates coated with poly-L-lysine

and incubated at 37°C for 1 h before MT-2 cells were added (10^5 cells in 100 □l added per well). Cell densities were reduced and the medium was replaced after 3 days of incubation when necessary. Neutralization was measured by staining viable cells with Finter's neutral red when cytopathic effects in control wells were >70% but less than 100%. Percentage protection was determined by calculating the difference in absorption (A_{540}) between test wells (cells + virus) and dividing this result by the difference in absorption between cell control wells (cells only) and virus control wells (virus only). Neutralizing titers are expressed as the reciprocal of the plasma dilution required to protect at least 50% of cells from virus-induced killing.

Results: Env was expressed at overall similar levels by plasmids containing either the secreted form of the antigen, but at a two-four-fold lower level by the sgp120-C3d expressing plasmids. Human 293T cells were transiently transfected with 2 µg of plasmid and both supernatants and cell lysates were assayed for gp120 using an antigen capture ELISA. The sgp120 constructs expressed from 450 to 800 ng per ml, whereas the 3C3d fusions expressed from 140 to 250 ng per ml. Approximately 90% of the Env protein was present in the supernatant for both sgp120 and sgp120-3C3d-DNA transfected cells (data not shown). The approximately 2-fold differences in the levels of expression of the different sgp120s is likely a reflection in differences in the Env genes as well as differences in the efficiency that the capture and detection antibodies recognized the different Envs.

Western blot analyses revealed sgp120 and sgp120-3C3d proteins of the

expected sizes. Using human patient polyclonal antisera, western blot analysis showed the expected broad band of 115-120 kD corresponding to gp120. A higher molecular weight band at ~240 kD was consistent with the projected size of the sgp120-3C3d fusion protein. Consistent with the antigen-capture assay, intense protein bands were present in the supernatants of cells transfected with sgp120-DNA, whereas less intense bands were present in the supernatants of cells transfected with sgp120-3C3d-DNA (data not shown). No evidence for the proteolytic cleavage of the sgp120-C3d fusion protein was seen by western analysis.

Antibody response to Env gp120 DNA immunizations: The sgp120-3C3d expressing DNA plasmids raised higher titers of ELISA antibody than the sgp120 DNA. BALB/c mice were vaccinated by DNA coated gold particles via gene gun with a 1μg dose inoculum. Mice were vaccinated at day 1 and then boosted at 4, 14, and 26 weeks with the same DNA given in the first immunization. When sera were assayed on gp120-IIIB -coated plates, mice vaccinated with the DNAs expressing the C3d fusion proteins had anti-Env antibodies 3-7 times higher then the amount of antibody raised by the counterpart sgp120 expressing plasmids. Among the C3d constructs, mice vaccinated with sgp120-(IIIB)-3C3d had the highest levels of antibody and mice vaccinated with sgp120-(ADA)-3C3d expressing DNA had the lowest levels of anti-Env antibodies. The temporal pattern for the appearance of anti-Env antibody revealed titers being boosted at each of the inoculations for all constructs tested.

Differences in the levels of the antibody raised by the different Envs

appeared to be determined by the specificity of the raised antibody. Using an alternative ELISA protocol, in which antibody was captured on the homologous Env, all of the C3d-fusions appeared to raise similar levels of antibody. In this assay, sheep anti-Env antibody was used to capture transiently produced sgp120 proteins. This assay revealed low, but similar levels of antibody raised by each of the sgp120-3C3d constructs. The lower levels of antibody detected in this assay are likely to reflect the levels of transfection-produced Env used to capture antibody being lower than in the assays using commercially produced IIIB gp120 to coat plates. As expected using either ELISA method, booster immunizations were necessary to achieve even the most modest antibody response.

Avidity of mouse Env antiserum: Sodium thiocyanate (NaSCN) displacement ELISAs demonstrated that the avidity of the antibody generated with sgp120-3C3d expressing DNA was consistently higher than that from sgp120-DNA vaccinated mice. Avidity assays were conducted on sera raised by sgp120-(IIIB) and sgp120-(IIIB)-3C3d because of the type specificity of the raised antisera and the commercial availability of the IIIB protein (but not the other proteins) for use as capture antigen. The avidity of specific antibodies to Env was compared by using graded concentrations NaSCN, a chaotropic agent, to disrupt antigen-antibody interaction. Results indicatedthat the antibody from sgp120-3C3d-DNA vaccinated mice underwent more rapid affinity maturation than antibody from sgp120-DNA vaccinated mice.

Env-3C3d expressing plasmids elicit modest neutralizing antibody: Neutralizing antibody studies performed on MT-2 cells detected higher titers of

neutralizing activity in the sera generated by the gp120-3C3d constructs than in the sera generated by the sgp120 constructs. Sera were tested against two syncytium inducing, IIIB (X4) and 89.6 (X4R5) viruses. Mice vaccinated with sgp120-3C3d expressing plasmids had very modest levels of neutralizing antibody to the homologous strain of HIV tested by the protection of MT-2 cells from virus-induced killing as measured by neutral red uptake. Titers of neutralizing antibody raised by the gp120-expressing DNAs were at the background of the assay.

The results of this study showed that fusions of HIV-1 Env to three copies of murine C3d enhanced the antibody response to Env in vaccinated mice. Mice vaccinated with any of the three DNA plasmids expressing sgp120 sequence had low or undetectable levels of antibody after 4 vaccinations (28 weeks post-prime). In contrast, mice vaccinated with DNA expressing the fusion of sgp120 and 3C3d proteins elicited a faster onset of antibody (3 vaccinations), as well as higher levels of antibodies.

In contrast to the enhancement of antibody titers and avidity maturation of antibodies to Env, the amount of neutralizing-antibody elicited in the vaccinated mice was low. Mice vaccinated with plasmids expressing sgp120 had low levels of neutralizing antibody that were only modestly increased in mice vaccinated with sp120-3C3d expressing plasmids. However, the levels of neutralizing antibodies did apparently increase after the fourth immunization. The poor titers of neutralizing antibody could have reflected an inherent poor ability of the sgp120-3C3d fusion protein to raise neutralizing antibody because of the failure

to adequately expose neutralizing epitopes to responding B cells. The intrinsic high backgrounds for HIV-1 neutralization assays in mouse sera also may have contributed to the poor neutralization titers.

The results demonstrate the effectiveness of C3d-fusions as a molecular adjuvant in enhancing antibody production and enhancing antibody maturation. In addition, the neutralizing antibody response to Env was modestly increased in mice vaccinated with C3d-fusion vaccines. Similar to results seen in Examples 17 and 18, using secreted versions of HA from measles and influenza viruses, C3d-enhanced antibody responses were achieved with plasmids expressing only half as much protein as plasmids expressing non-fused sgp120.

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CLAIMS

What is claimed is:

1. A vector comprising:

   a termination sequence encoding for the lambda T0 terminator;

   a prokaryotic origin of replication;

   a selectable marker gene; and

   a eukaryotic transcription cassette comprising a vaccine insert encoding one or more immunogens derived from a pathogen.

2. The vector of Claim 1, wherein the pathogen is a viral pathogen.

3. The vector of Claim 1, wherein the viral pathogen is selected from the group consisting of HIV, measles, influenza, polio and rubella.

4. The vector of Claim 1, wherein the one or more immunogens is selected from the group consisting of HIV Gag, HIV gp120 HIV Pol, HIV Env, HIV VLP, measles fusion protein, measles hemagglutinin, measles nucleoprotein, influenza hemagglutinin, mutants thereof, and subsequences thereof.

5. The vector of Claim 1, wherein the one or more immunogens are further selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, and HIV VLP, mutants thereof, and subsequences thereof.

6. The vector according to Claim 1, wherein the vaccine insert encoding one or more immunogen or immunogens further comprises at least one C3d gene.

7. A method of immunizing or treating a patient, comprising the step of administering a therapeutically effective amount of a physiologically acceptable composition, comprising the vector of Claim 1.

8. The method of Claim 7, further comprising the step of subsequently administering a therapeutically effective amount of a composition comprising a recombinant pox virus vector expressing one or more immunogens selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, measles fusion protein, measles hemagglutinin, measles nucleoprotein, influenza hemagglutinin, mutants thereof, and subsequences thereof.

9. The method of Claim 7, wherein the one or more immunogens is selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, mutants thereof, and subsequences thereof.

10. The method of Claim 7, further comprising subsequently administering a therapeutically effective amount of a recombinant pox virus vector

expressing one or more immunogens selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, measles fusion protein, measles hemagglutinin, measles nucleoprotein, influenza hemagglutinin, mutants thereof, and subsequences thereof.

11. The method of Claim 7, wherein the vector expresses one or more immunogens selected from the group consisting of HIV Gag, HIV gp120. HIV Pol, HIV Env, HIV VLP, mutants thereof, and subsequences thereof.

12. A vector comprising the DNA sequence SEQ ID NO: 1.

13. The vector of Claim 12, wherein the vector further comprises a vaccine insert encoding one or more immunogens selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, measles fusion protein, measles hemagglutinin, measles nucleoprotein, influenza selected hemagglutinin, influenza transmembrane hemagglutinin mutants thereof, and subsequences thereof; and optionally at least one C3d gene.

14. The vector of Claim 12, wherein the one or more immunogens is selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, mutants thereof, and subsequences thereof.

15. A vector comprising the DNA sequence SEQ ID NO: 2.

16. The vector of Claim 15, wherein the vector further comprises a vaccine insert encoding one or more immunogens selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, measles fusion protein, measles hemagglutinin, measles nucleoprotein, influenza selected hemagglutinin, influenza transmembrane hemagglutinin mutants thereof, and subsequences thereof; and optionally at least one C3d gene.

17. The vector of Claim 15, wherein the one or more immunogens is selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, mutants thereof, and subsequences thereof.

18. A vector comprising the DNA sequence SEQ ID NO: 3.

19. The vector of Claim 18, wherein the vector further comprises a vaccine insert encoding one or more immunogens selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, measles fusion protein, measles hemagglutinin, measles nucleoprotein, influenza selected hemagglutinin, influenza transmembrane hemagglutinin mutants thereof, and subsequences thereof; and optionally at least one C3d gene.

20. The vector of Claim 18, wherein the one or more immunogens is selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, mutants thereof, and subsequences thereof.

21. A vector comprising the DNA sequence SEQ ID NO: 4.

22. The vector of Claim 21, wherein the vector further comprises a vaccine insert encoding one or more immunogens selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, measles fusion protein, measles hemagglutinin, measles nucleoprotein, influenza selected hemagglutinin, influenza transmembrane hemagglutinin mutants thereof, and subsequences thereof; and optionally at least one C3d gene.

23. The vector of Claim 21, wherein the one or more immunogens is selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, mutants thereof, and subsequences thereof.

24. A vector comprising the DNA sequence SEQ ID NO: 5.

25. The vector of Claim 24, wherein the vector further comprises a vaccine insert encoding one or more immunogens selected from the group

consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, measles fusion protein, measles hemagglutinin, measles nucleoprotein, influenza selected hemagglutinin, influenza transmembrane hemagglutinin mutants thereof, and subsequences thereof; and optionally at least one C3d gene.

26. The vector of Claim 24, wherein the one or more immunogens is selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, mutants thereof, and subsequences thereof.

27. A method of immunizing or treating a patient comprising administering a therapeutically effective amount of a physiologically acceptable composition comprising a vector wherein the vector is a nucleic acid comprising a sequence selected from SEQ ID NOS: 1, 2, 3, 4 and 5, and wherein administration of the therapeutically effective amount of the composition is by an intramuscular or intradermal route.

28. The method of Claim 27, wherein the vector expresses one or more immunogens selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, measles fusion protein, measles hemagglutinin, measles nucleoprotein, influenza hemagglutinin, mutants thereof, and subsequences thereof; and optionally at least one C3d gene.

29. The method of Claim 27, wherein the vector expresses one or more immunogens selected from the group consisting of HIV Gag, HIV gp120. HIV Pol, HIV Env, HIV VLP, mutants thereof, and subsequences thereof.

30. The method of Claim 27, further comprising subsequently administering a therapeutically effective amount of a recombinant pox virus vector expressing one or more immunogens selected from the group consisting of HIV Gag, HIV gp120, HIV Pol, HIV Env, HIV VLP, measles fusion protein, measles hemagglutinin, measles nucleoprotein, influenza hemagglutinin, mutants thereof, and subsequences thereof.

31. The method of Claim 27, wherein the vector expresses one or more immunogens selected from the group consisting of HIV Gag, HIV gp120. HIV Pol, HIV Env, HIV VLP, mutants thereof, and subsequences thereof.

32. A method of immunizing or treating a patient in need thereof comprising administering a therapeutically effective amount of a composition comprising the vector of Claim 29.

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Fig. 1

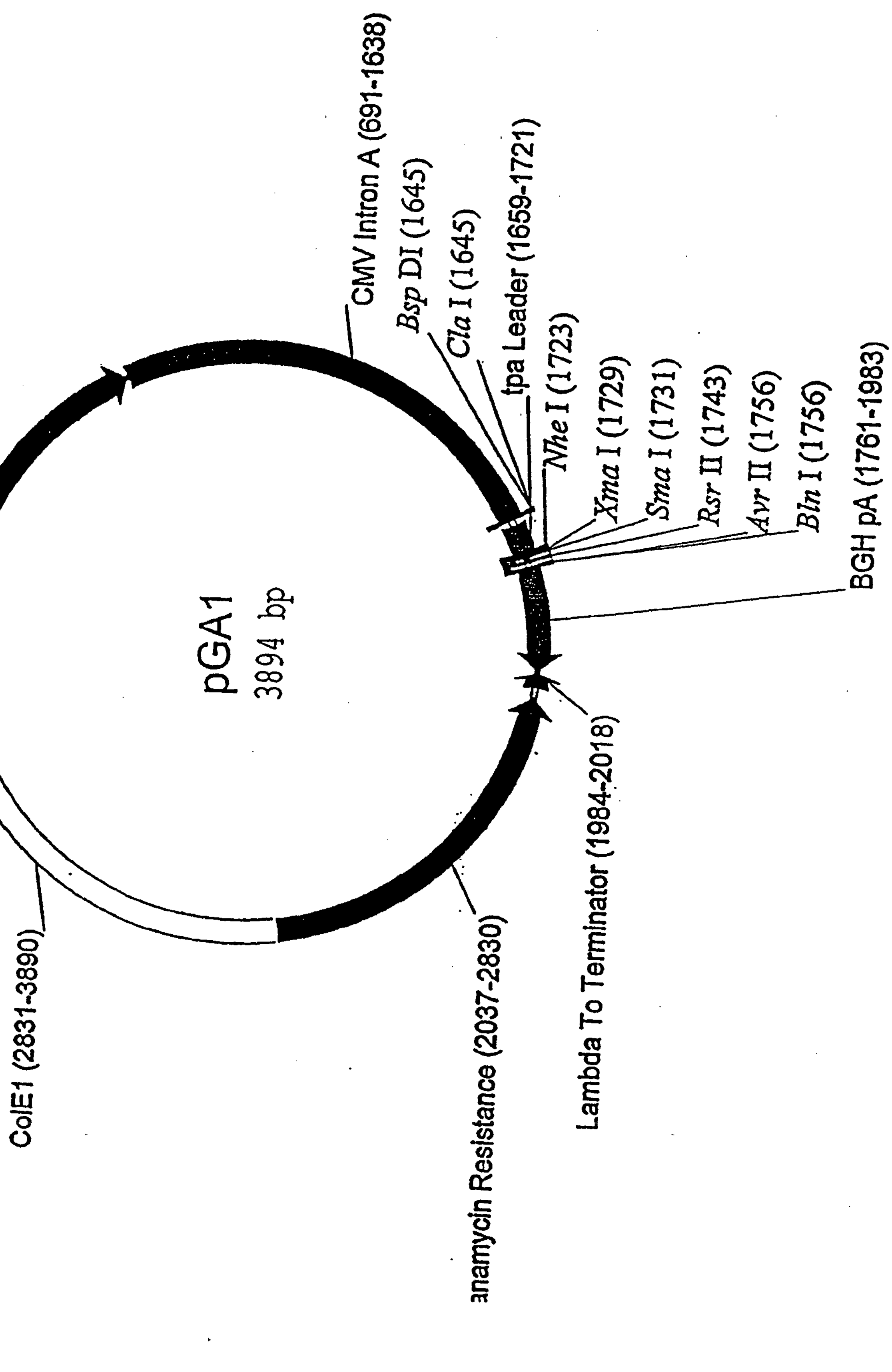
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Fig. 2

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                                  Kanamycin Resistance                                                                       Col

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                                  ColE1

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Tuesday, December 21, 1999 7:23 PM
pGA1 Final Version Map.MPD (1 > 3894) Site and Sequence

Page 4

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ColE1

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ColE1

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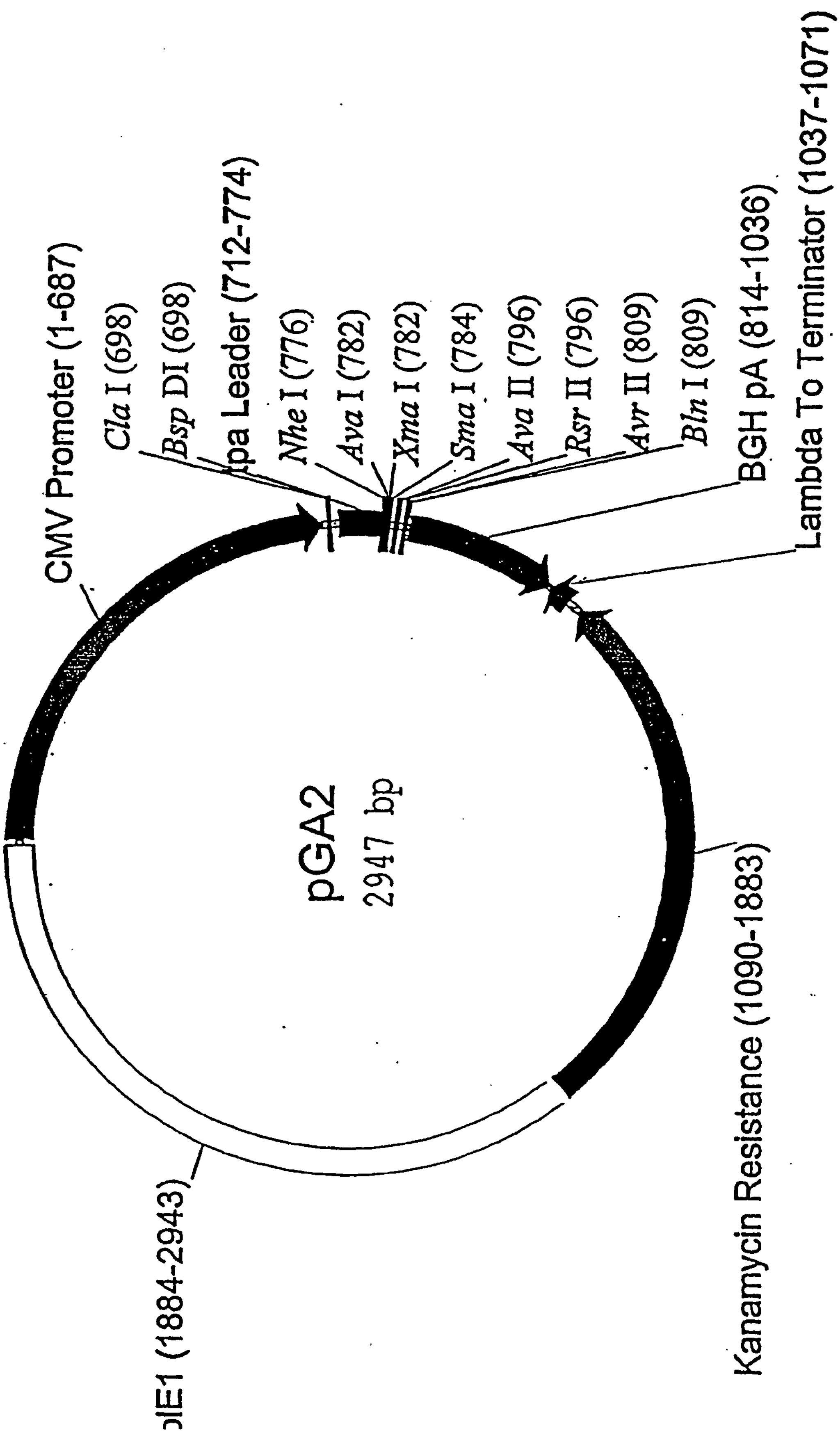
ColE1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Nhe I (776)
Ava I (782)
Xma I (782)
Sma I (784)
Ava II (796)
Rsr II (796)
Avr II (809)
Bln I (809)
BGH pA (814-1036)
Lambda To Terminator (1037-1071)
pGA2
2947 bp
Kanamycin Resistance (1090-1883)
IE1 (1884-2943)

Fig. 3

Fig. 7

pGA2 Final Version Map.MPD (1 > 2947) Site and Sequence
Enzymes : 9 of 518 enzymes (Filtered)
Settings : Circular, Subrange Context, Certain Sites Only, Standard Genetic Code

Page

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CMV Promoter

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CMV Promoter

                     BspD I
                     Cla I                                                                                  Nhe I  Ava I Xma I Sma I  Ava II Rsr II  Avr II
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CMV Promot>
                              M  D  A  M  K  R  G  L  C  C  V  L  L  L  C  G  A  V  F  V  S
                              tpa Leader

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BGH pA
```

pGA2 Final Version Map.MPD (1 > 2947) Site and Sequence

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BGH pA                                             Lambda To Terminator

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Kanamycin Resistance

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Kanamycin Resistance

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Kanamycin Resistance                                                                                                                    ColE1

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                                                                                                                                        2025
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ColE1
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pGA2 Final Version Map.MPD (1 > 2947) Site and Sequence


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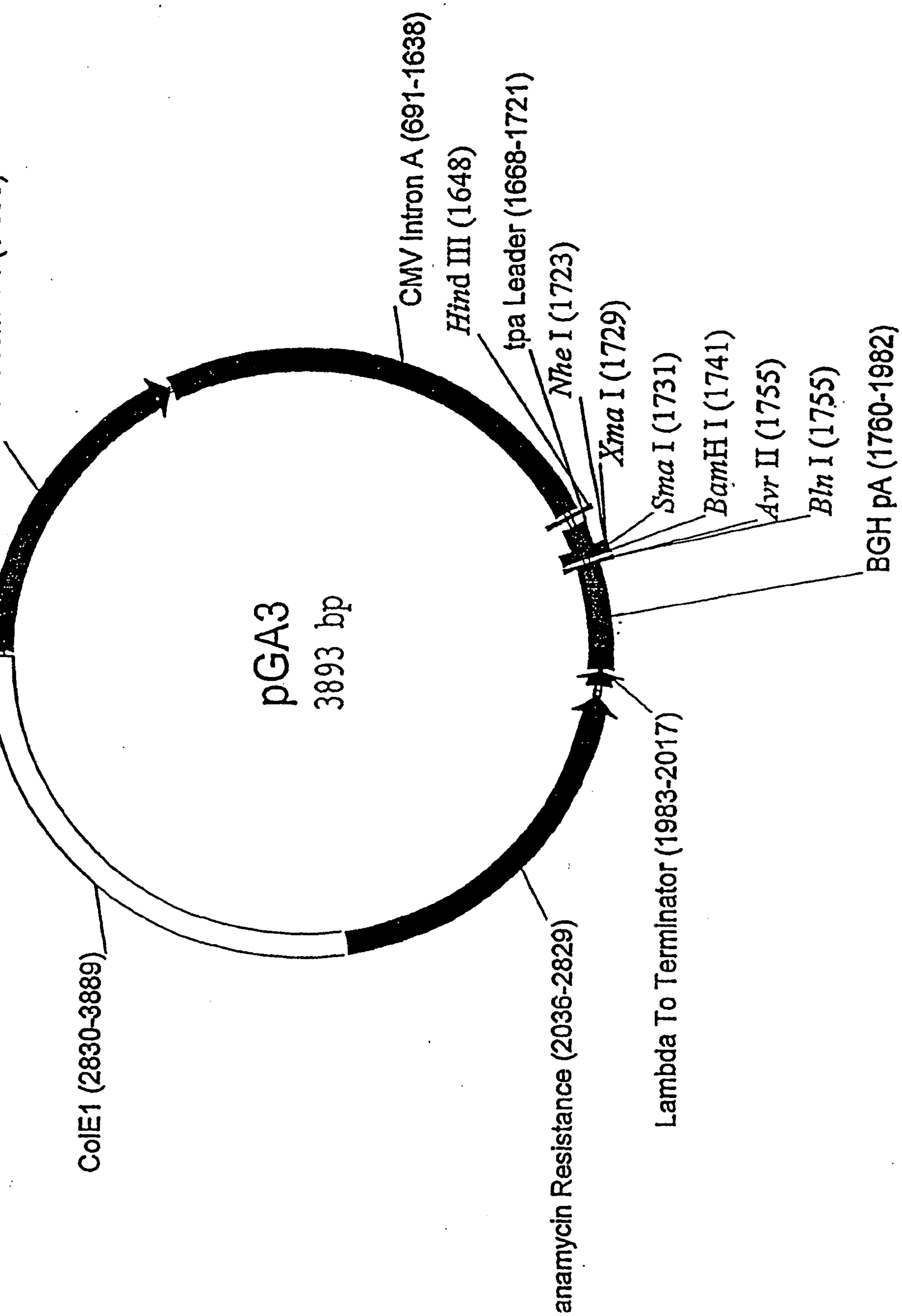
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Avr II (1755)
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ColE1 (2830-3889)

Fig. 5

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                  Hind III                                                                         Nhe I  Xma I  Sma I  BamH I   Avr II
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                                                     tpa Leader

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BGH pA                                                              Lambda To Terminator

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Kanamycin Resistance
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Kanamycin Resistance

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Kanamycin Resistance

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Kanamycin Resistance

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Kanamycin Resistance ColE1

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ColE1

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ColE1

Amount of Total IgG Antibodies
Elicited from Gene GunVaccinated Mice

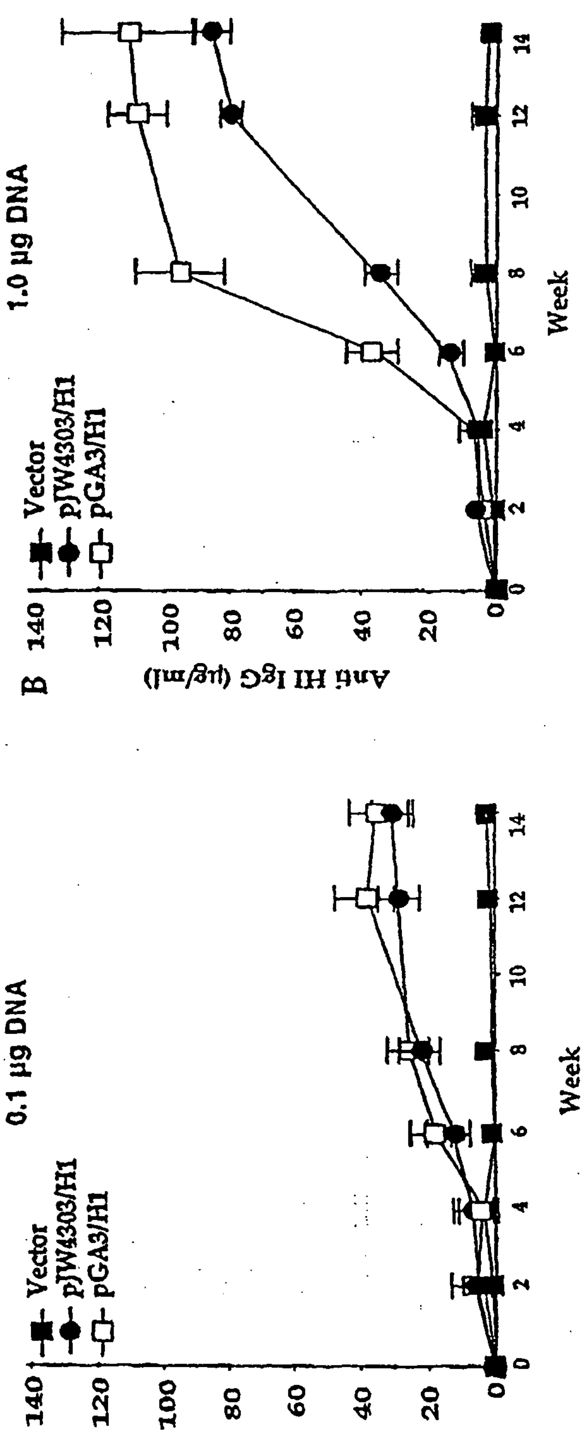
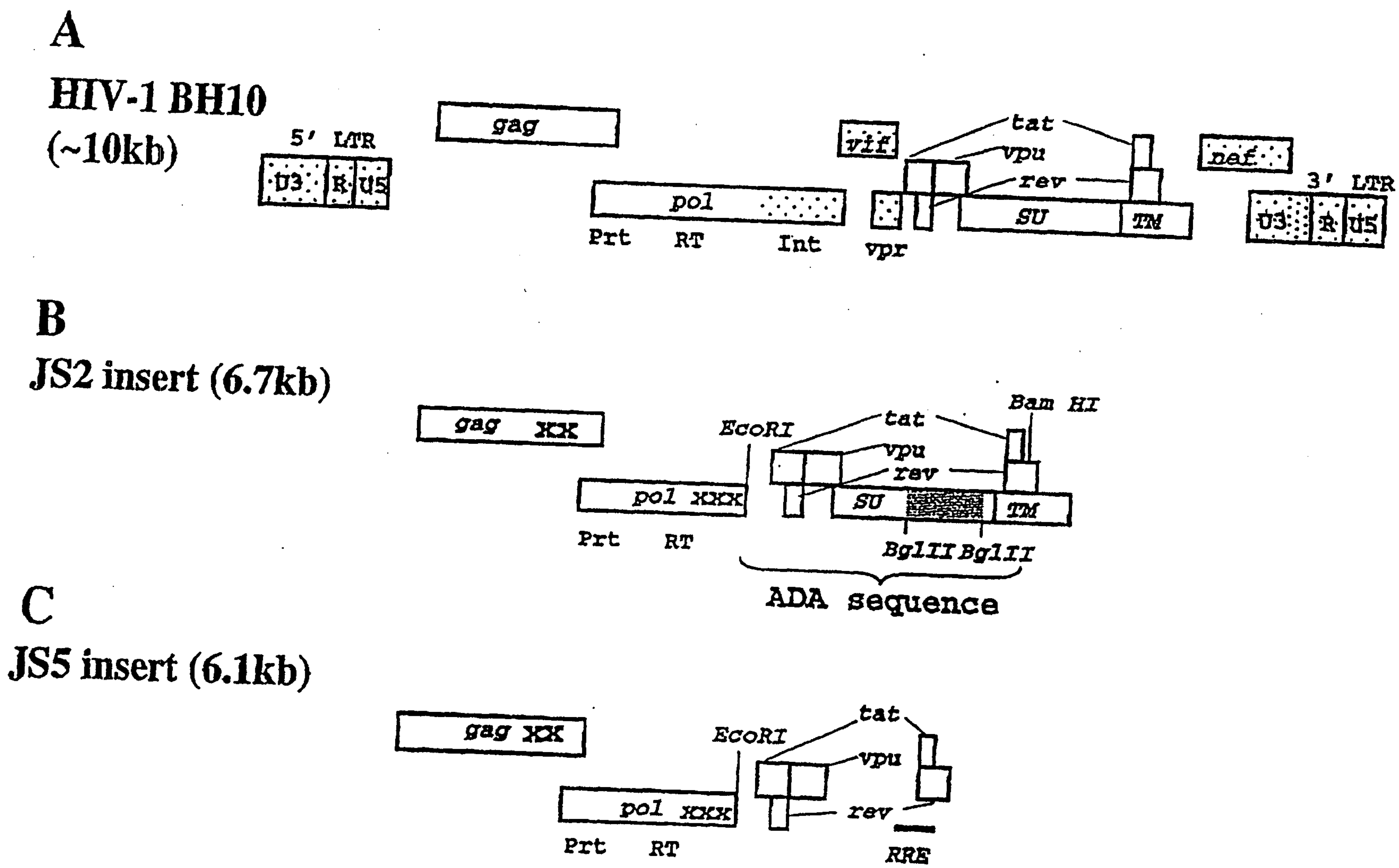


Fig. 7

Fig. 8


A
HIV-1 BH10
(~10kb)
5' LTR
U3
R
U5
gag
pol
Prt
RT
Int
vif
vpr
tat
vpu
rev
SU
TM
nef
3' LTR
U3
R
U5
B
JS2 insert (6.7kb)
gag XX
pol XXX
Prt
RT
EcoRI
tat
vpu
rev
Bam HI
SU
TM
BglII BglII
ADA sequence
C
JS5 insert (6.1kb)
gag XX
pol XXX
Prt
RT
EcoRI
tat
vpu
rev
RRE

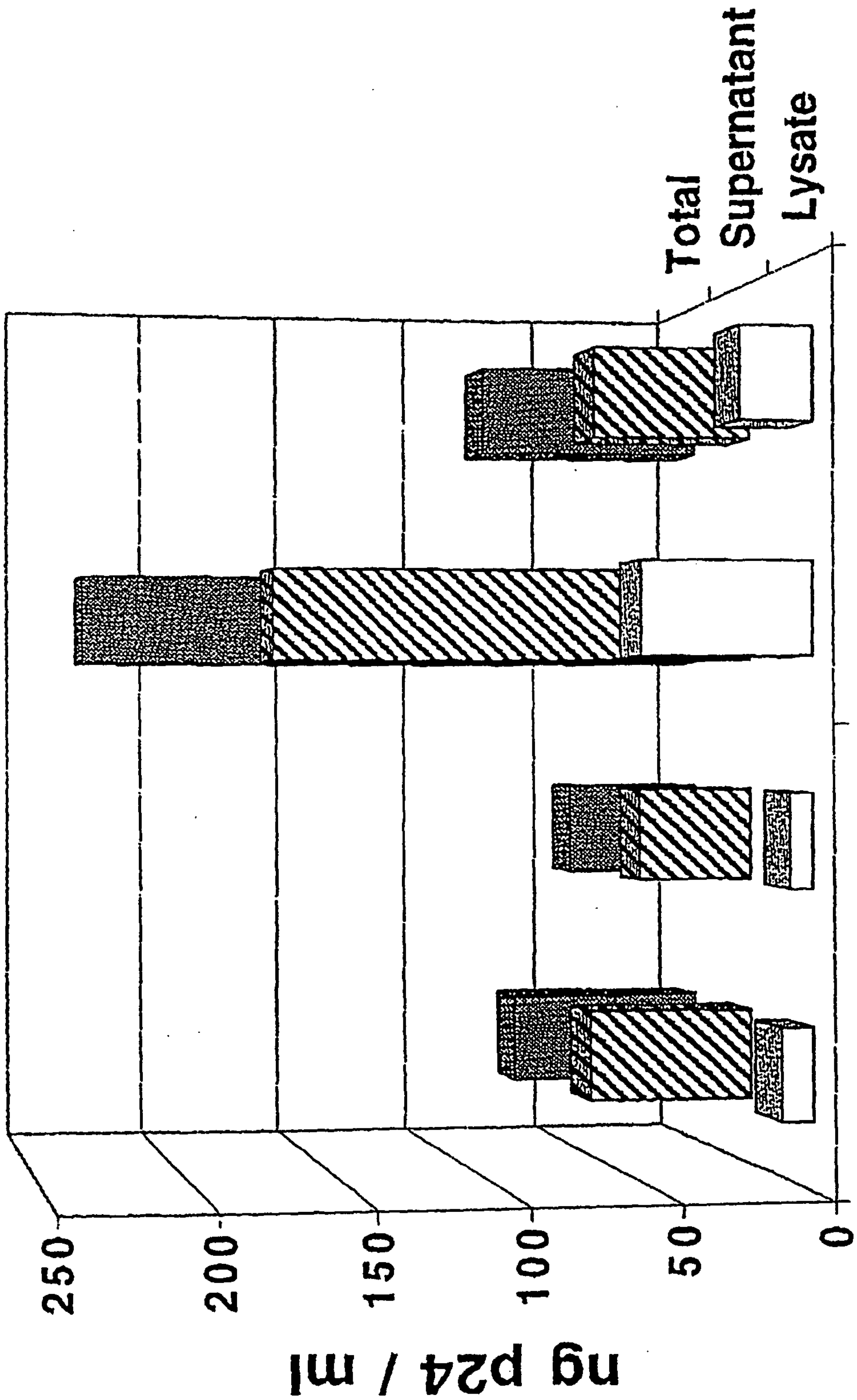

ng p24 / ml
250
200
150
100
50
0
ADA wt
IIIb wt
ADA VLP
dPOL
Total
Supernatant
Lysate

Fig. 9 A

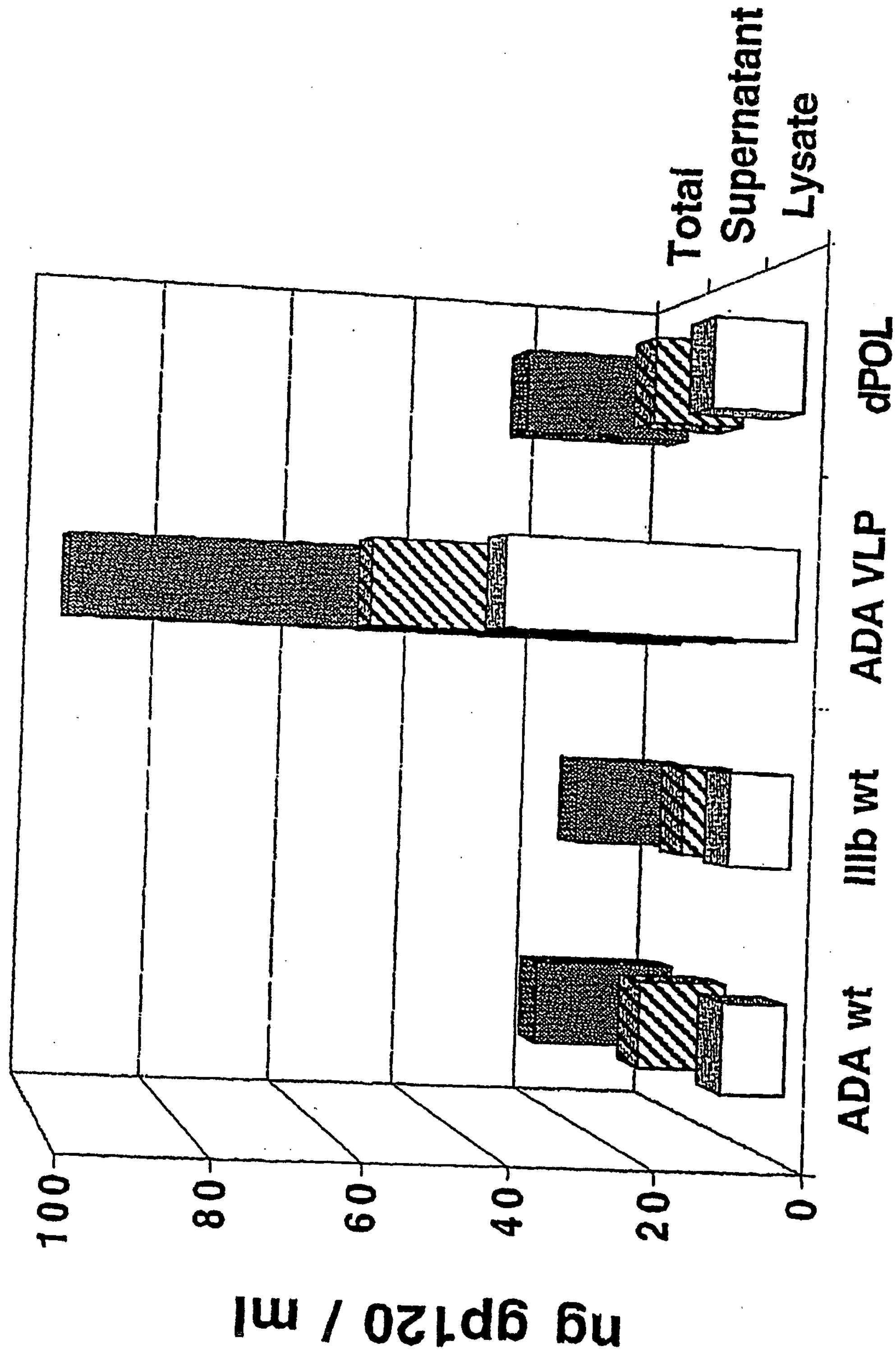
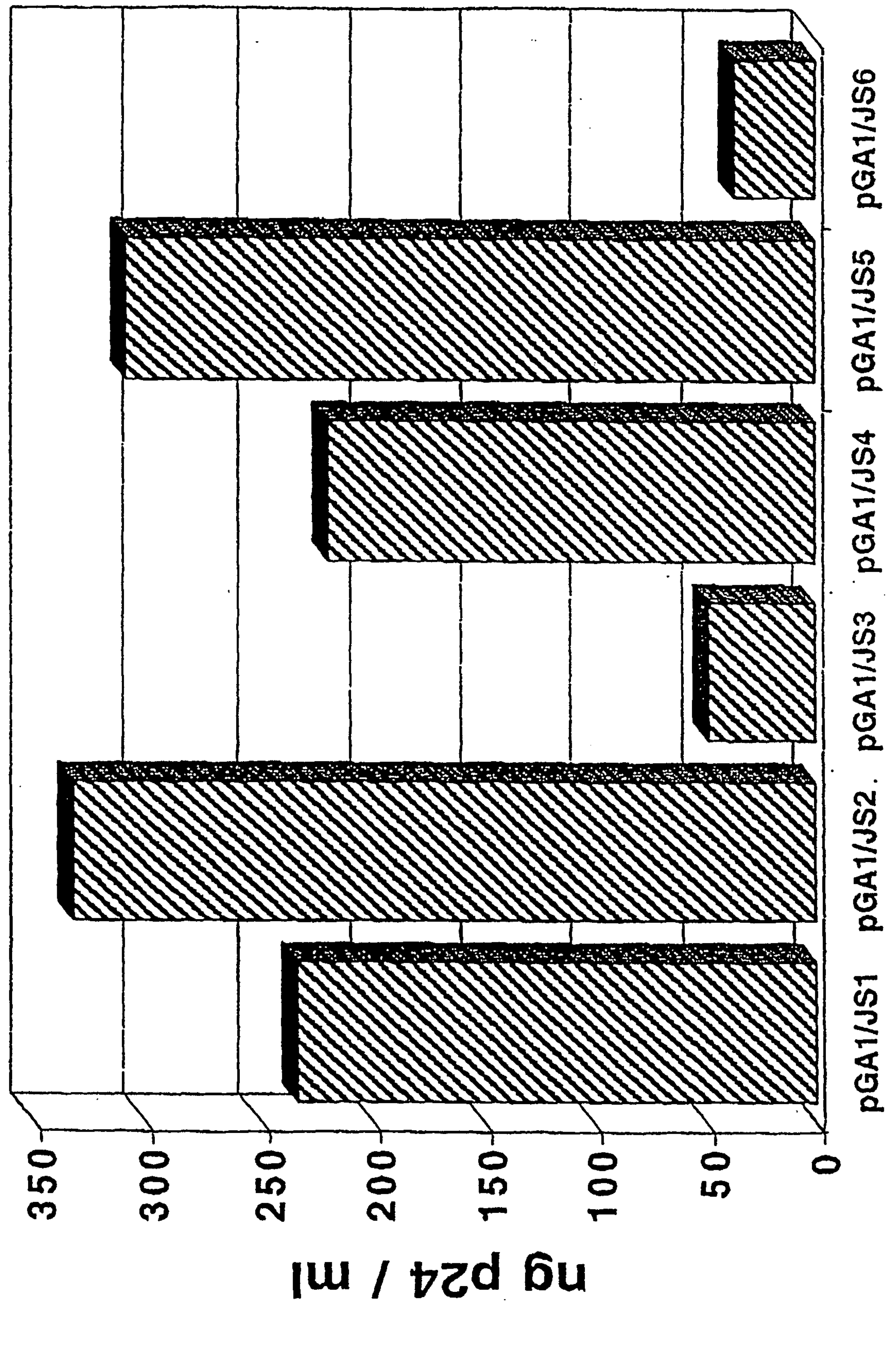

ng gp120 / ml
0
20
40
60
80
100
ADA wt
IIIb wt
ADA VLP
dPOL
Total
Supernatant
Lysate

Fig. 9 B


ng p24 / ml
0
50
100
150
200
250
300
350
pGA1/JS1
pGA1/JS2
pGA1/JS3
pGA1/JS4
pGA1/JS5
pGA1/JS6

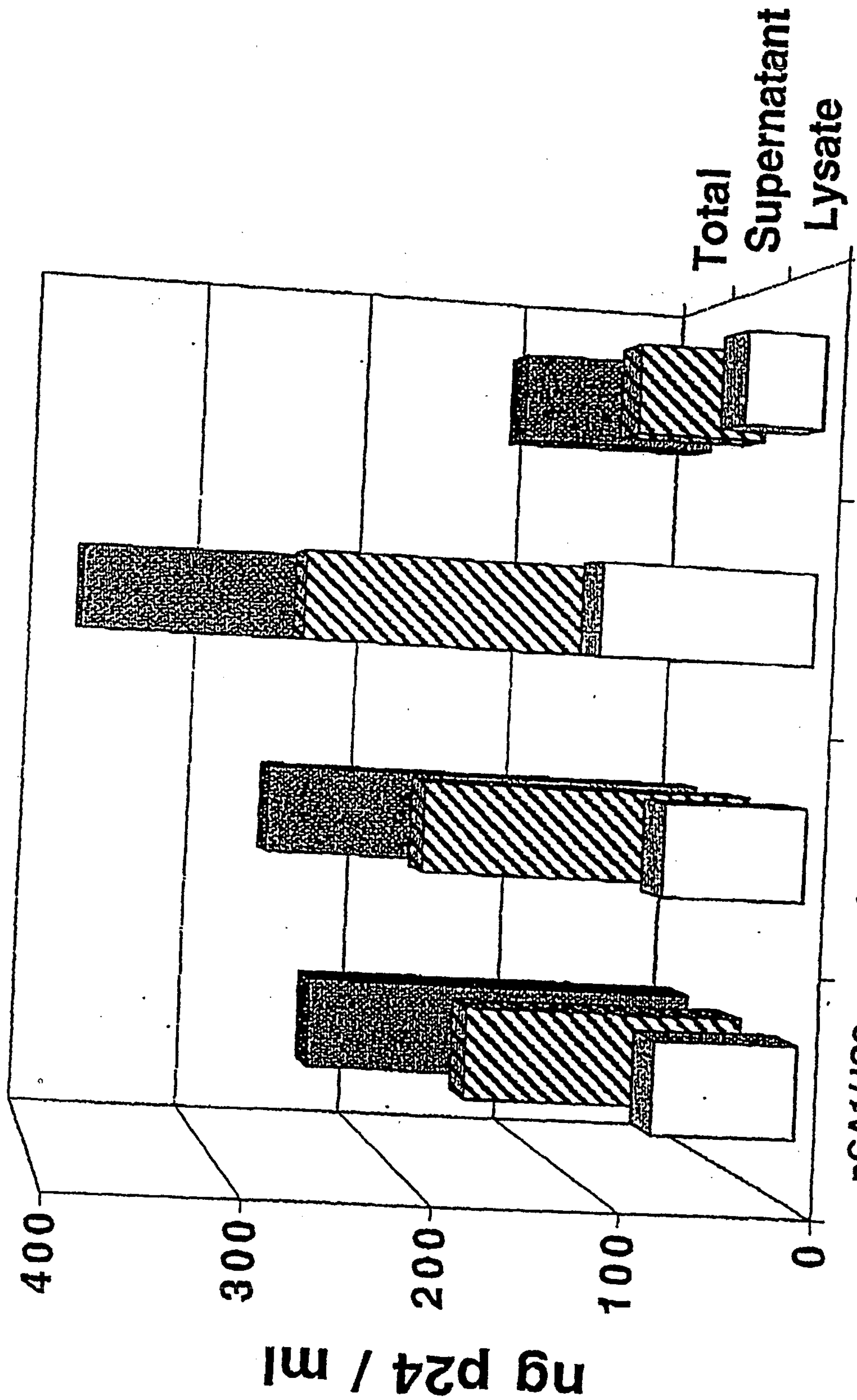

Total
Supernatant
Lysate
pGA1/JS2
pGA2/JS2
pGA1/JS5
pGA2/JS5
0
100
200
300
400
ng p24 / ml

Fig. 11 A

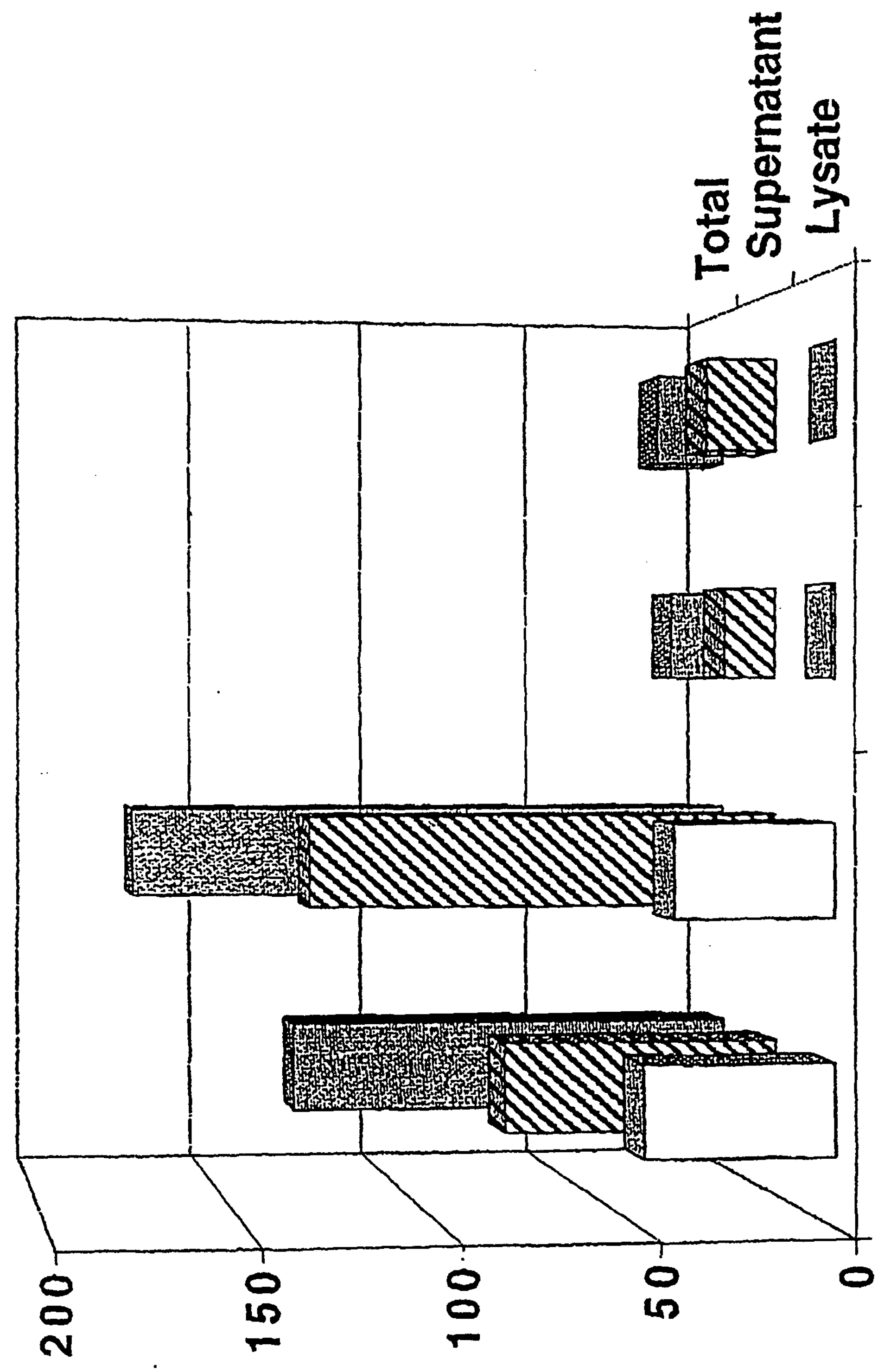
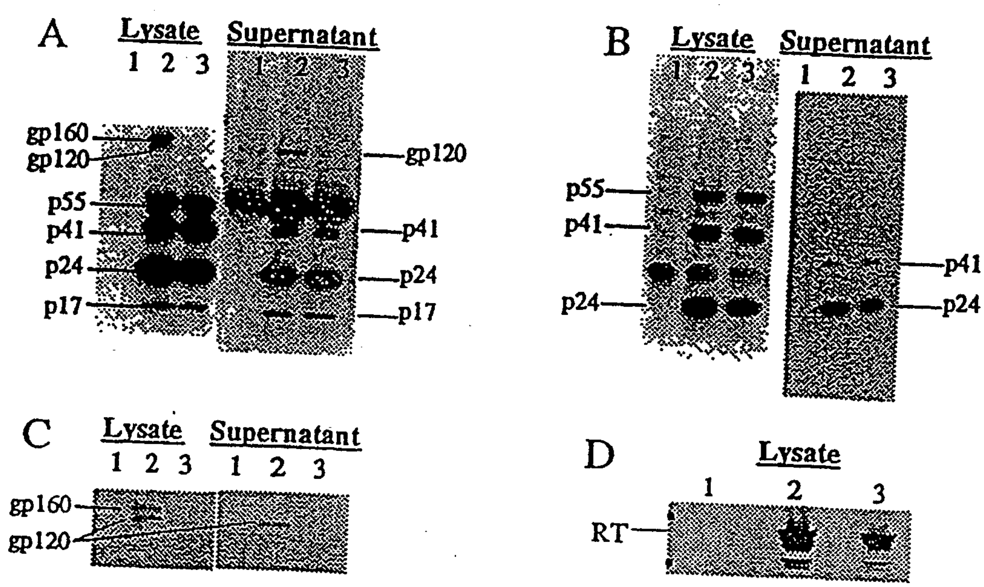

Total
Supernatant
Lysate
ng gp120 / ml
200
150
100
50
0
pGA1/JS2
pGA2/JS2
pGA1/JS5
pGA2/JS5

Fig. 11B

Fig. 12



Western blots of cell lysates and tissue culture supernatants from 293T cells transfected with: 1) Mock 2) pGA2/JS2 3) pGA1/JS5 Primary antibody: A) pooled anti-HIV Ig from infected patients B) anti-p24 C) anti-gp120 D) anti-RT.

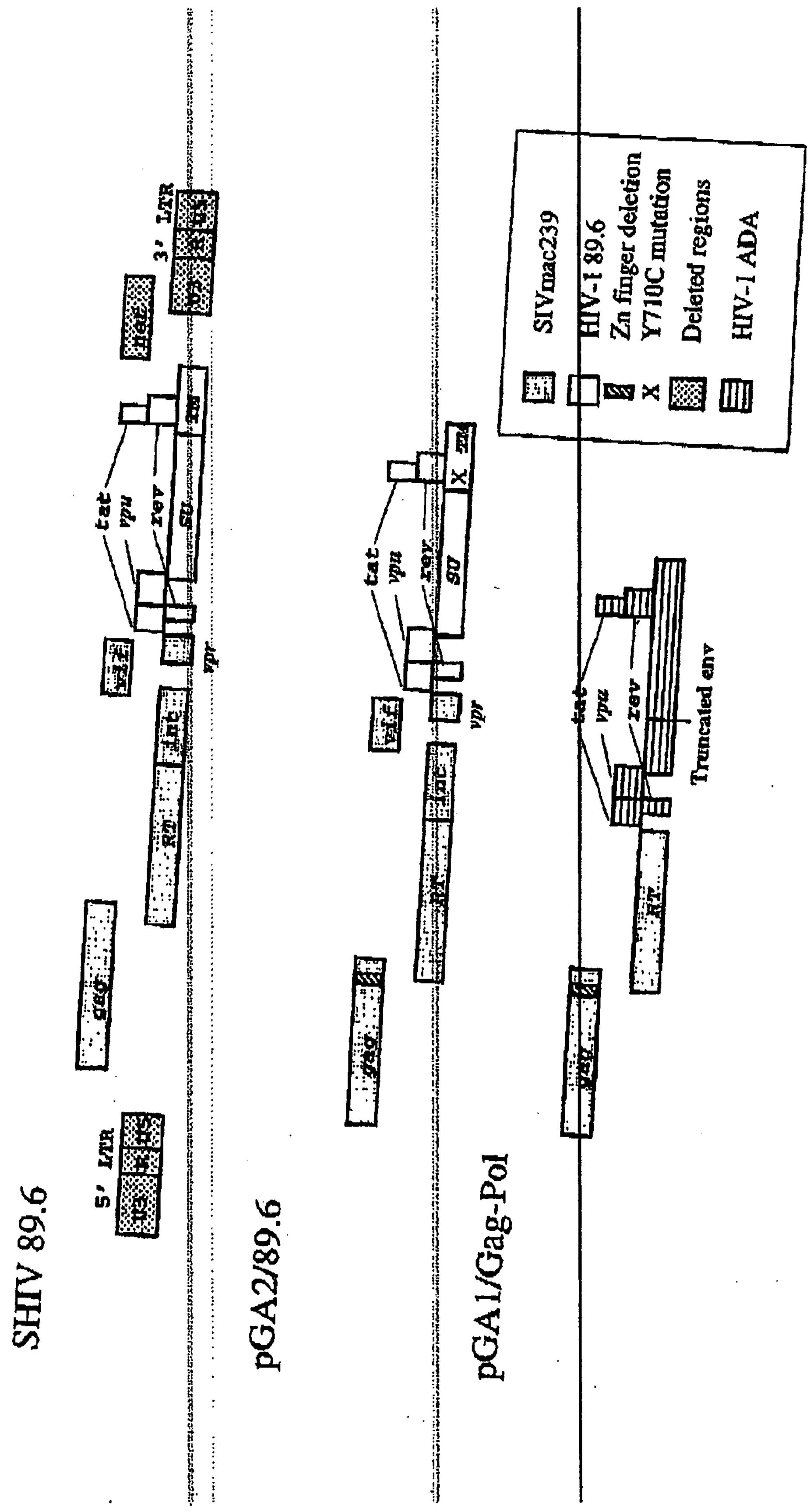

SHIV 89.6
5' LTR
gag
RT
Int
vif
tat
vpu
rev
vpr
SU
TM
nef
3' LTR
pGA2/89.6
pGA1/Gag-Pol
Truncated env
SIVmac239
HIV-1 89.6
Zn finger deletion
Y710C mutation
Deleted regions
HIV-1 ADA

FIGURE 13

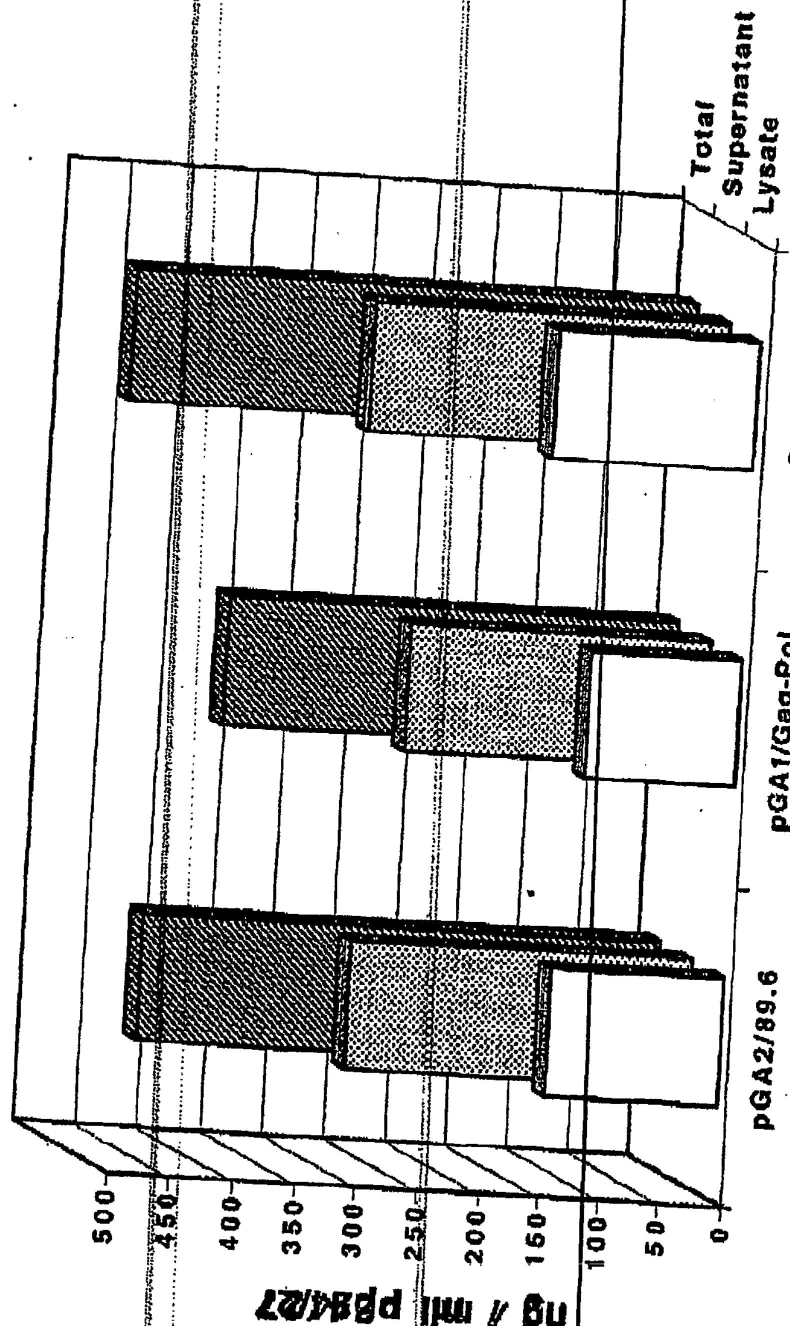

Total
Supernatant
Lysate
pGA2/89.6
pGA1/Gag-Pol
pGA2/JS2
0
50
100
150
200
250
300
350
400
450
500

FIGURE 14

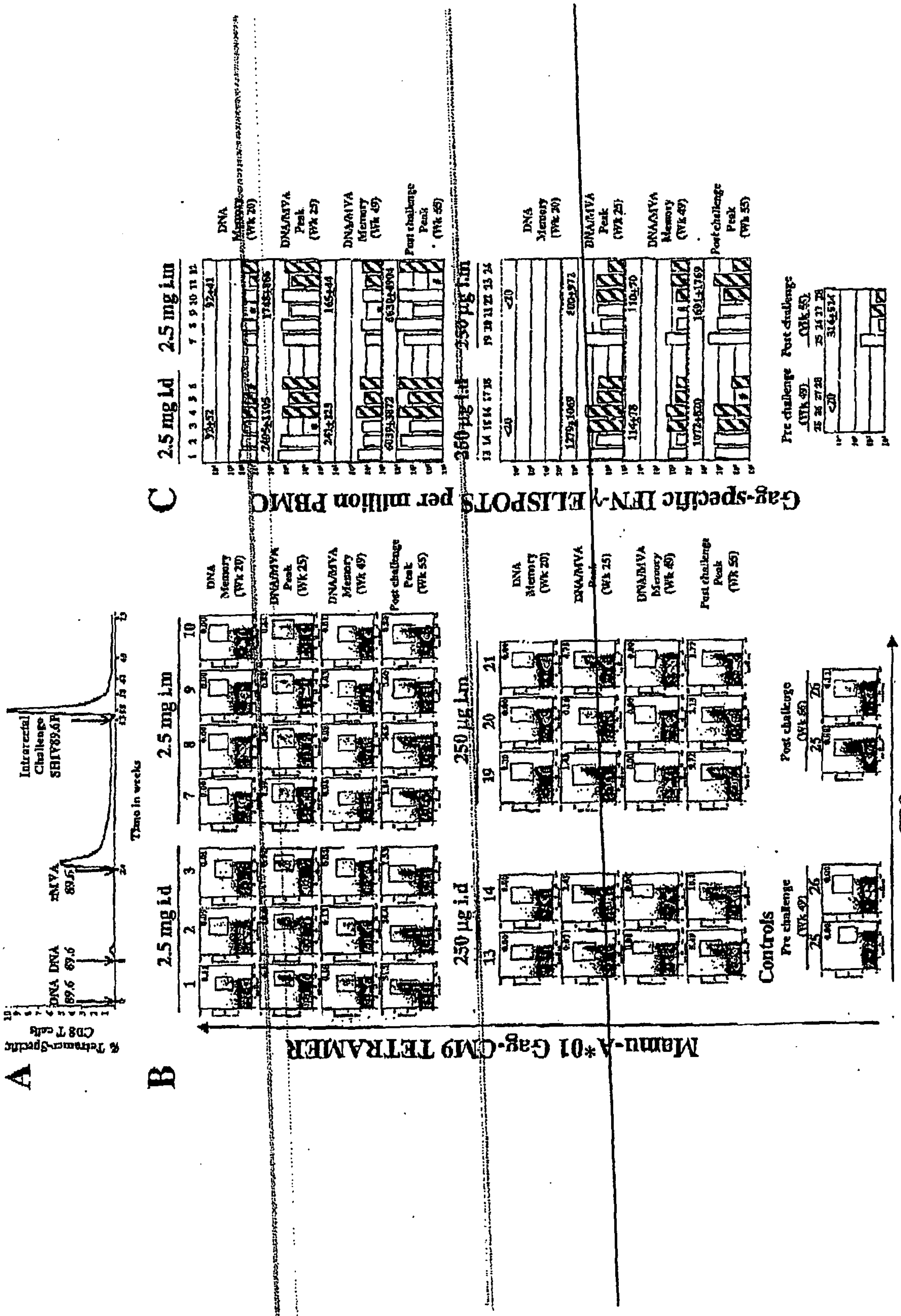
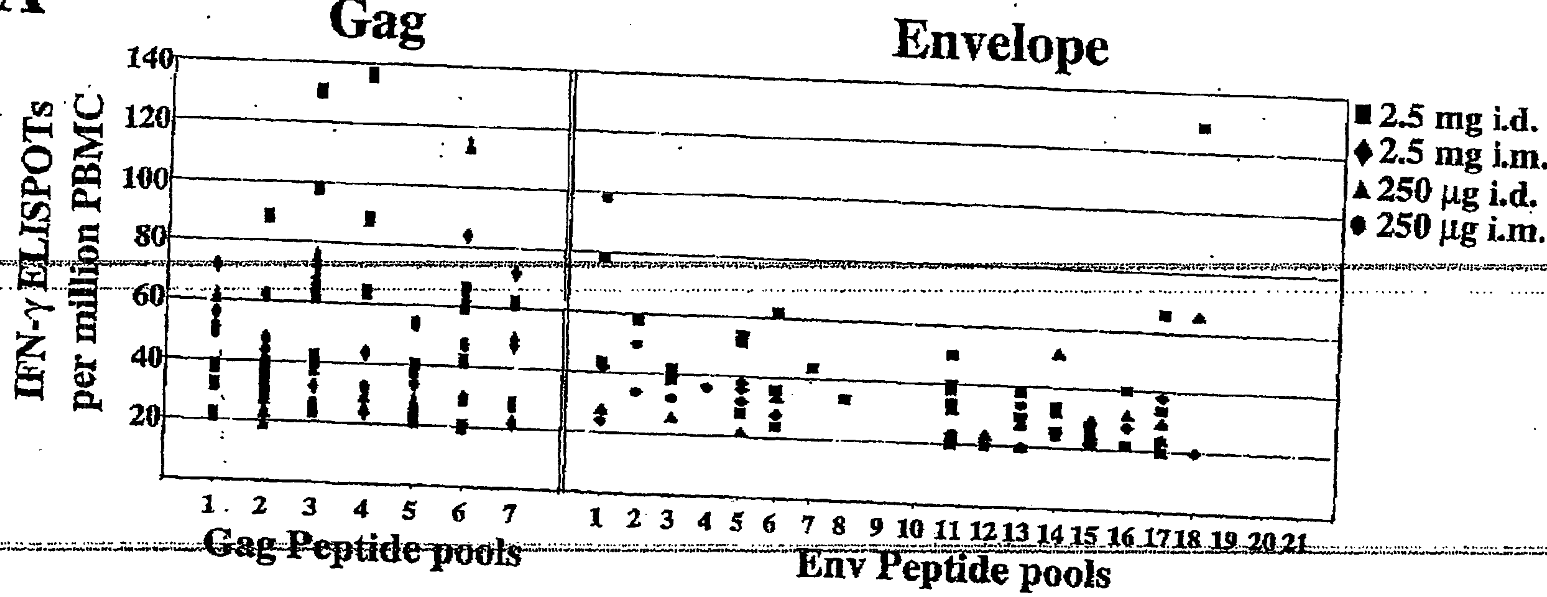

A
B
C
Mamu-A*01 Gag-CM9 TETRAMER
CD8
Gag-specific IFN-γ ELISPOTS per million PBMC
2.5 mg i.d
2.5 mg i.m
250 μg i.d
250 μg i.m
Controls
Pre challenge
Post challenge
DNA Memory (Wk 20)
DNA/MVA Peak (Wk 25)
DNA/MVA Memory (Wk 49)
Post challenge Peak (Wk 55)
Intrarectal Challenge SHIV89.6P
Time in weeks

FIGURE 15

A



B

DNA Prime	Height of ELISPOTs			Breadth of ELISPOTs		
	Gag	Env	Total	Gag	Env	Total
2.5 mg, i.d.	285±130	224±76	509±137	4.5±1.2	5.3±2.0	9.8±2.4
2,5 mg, i.m	203±97	83±44	286±73	4.4±1.8	2.8±1.3	7.2±1.1
250 µg, i.d	104±113	101±77	205±183	2.2±2.5	3.5±2.4	5.7±4.5
250 µg, i.m	76±99	84±110	160±207	1.7±2.0	2.3±2.9	4.0±4.7
Control DNA	0	0	0	0	0	0

FIG. 16

J. 7

... map (1 - 5048) Site and Sequence
nes : All 480 enzymes (No Filter)
ge : Circular, Subrange Context, Certain Sites Only, Standard Genetic Code

```
GATGCAGGACTCGGCTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGGTGGGTACGCCAAAAATTTTGACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGATCGA 150
                                                                                                  M G A R A S V L S G G E L D R
                                                                                                  ------------ gag ------------
GAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAACATATAGTATGGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACATCAGAAGGCTGTAGACAAATACTGGGACAGCTACAA 300
E K I R L R P G G K K K Y K L K H I V W A S R E L E R F A Y N P G L L E T S E G C R Q I L G Q L Q
------------------------------------------------ gag ------------------------------------------------
TCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATATAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAAGCA 450
S L Q T G S E E L R S L Y N T V A T L Y C V H Q R I E I K D T K E A L D K I E E E Q N K S K K K A
------------------------------------------------ gag ------------------------------------------------
CAAGCAGCAGCTGACACAGGACACAGCAGTCAGGTCAGCCAAAATTACCCTATAGTGCAGAACATCCAGGGGCAAATGGTACATCAGGCCATATCACCTAGAACTTTAAATGCATGGGTAAAAGTAGTAGAAGAGAAGGCTTTCAGC 600
Q A A A D T G H S S Q V S Q N Y P I V Q N I Q G Q M V H Q A I S P R T L N A W V K V V E E K A F S
------------------------------------------------ gag ------------------------------------------------
GAAGTAATACCCATGTTTTCAGCATTATCAGAAGGAGCCACCCCACAAGATTTAAACACCATGCTAAACACAGTGGGGGGACATCAAGCAGCCATGCAAATGTTAAAAGAGACCATCAATGAGGAAGCTGCAGAATGGGATAGAGTA 750
E V I P M F S A L S E G A T P Q D L N T M L N T V G G H Q A A M Q M L K E T I N E E A A E W D R V
------------------------------------------------ gag ------------------------------------------------
CAGTGCATGCAGGGCCTATTGCACCAGGCCAGATGAGAGAACCAAGGGGAAGTGACATAGCAGGAACTACTAGTACCCTTCAGGAACAAATAGGATGGATGACAAATAATCCACCTATCCCAGTAGGAGAAATTTATAAAAGATGG 900
P V H A G P I A P G Q M R E P R G S D I A G T T S T L Q E Q I G W M T N N P P I P V G E I Y K R W
------------------------------------------------ gag ------------------------------------------------
ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTACCAGCATTCTGGACATAAGACAAGGACCAAAAGAACCTTTTAGAGACTATGTAGACCGGTTCTATAAAACTCTAAGAGCCGAGCAAGCTTCACAGGAGGTAAAAAAT 1050
I L G L N K I V R M Y S P T S I L D I R Q G P K E P F R D Y V D R F Y K T L R A E Q A S Q E V K N
------------------------------------------------ gag ------------------------------------------------
ATGACAGAAACCTTGTTGGTCCAAAATGCGAACCCAGATTGTAAGACTATTTTAAAAGCATTGGGACCAGCGGCTACACTAGAAGAAATGATGACAGCATGTCAGGGAGTAGGAGGACCCGGCCATAAGGCAAGAGTTTTGGCTGAA 1200
M T E T L L V Q N A N P D C K T I L K A L G P A A T L E E M M T A C Q G V G G P G H K A R V L A E
------------------------------------------------ gag ------------------------------------------------
ATGAGCCAAGTAACAAATACAGCTACCATAATGATGCAGAGAGGCAATTTTAGGAACCAAAGAAAGATGGTTAAGACCTTCAATAGCGCCAAAGAAGGGCACACAGCCAGAAATTGCAGGGCCCCTAGGAAAAAGGGCAGCTGGAAA 1350
M S Q V T N T A T I M M Q R G N F R N Q R K M V K S F N S G K E G H T A R N C R A P R K K G S W K
------------------------------------------------ gag ------------------------------------------------
GGAAAGGAAGGACACCAAATGAAAGATTGTACTGAGAGACAGGCTAATTTTTTAGGGAAGATCTGGCCTTCCTACAAGGGAAGGCCAGGGAATTTTCTTCAGAGCAGACCAGAGCCAACAGCCCCACCATTTCTTCAGAGCAGACCA 1600
G K E G H Q M K D C T E R Q A N F L G K I W P S Y K G R P G N F L Q S R P E P T A P P F L Q S R P
------------------------------------------------ gag ------------------------------------------------
                                F F R E D L A F L Q G K A R E F S S E Q T R A N S P T I S S E Q T
                                ---------------------------------- pol ----------------------------------
```

Map (1 > 9545) Site and Sequence Page 2

```
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 P  T  A  P  P  E  E  S  F  R  S  G  V  E  T  T  T  P  P  Q  K  Q  E  P  I  D  K  E  L  Y  P  L  T  S  L  R  S  L  F  G  N  D  P  S  S  Q  .
                                                                   gag
 A  N  S  P  T  R  R  E  L  Q  V  W  G  R  D  N  N  S  P  S  E  A  G  A  D  R  Q  G  T  V  S  F  N  F  P  Q  I  T  L  W  Q  R  P  L  V  T  I  K  I
                                                                   pol

GGGCAACTAAAGGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGAGTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAAGACAGTATGATCAGATACTCATAGAAA 1800
 G  Q  L  K  E  A  L  L  D  T  G  A  D  D  T  V  L  E  E  M  S  L  P  G  R  W  K  P  K  M  I  G  G  I  G  G  F  I  K  V  R  Q  Y  D  Q  I  L  I  E
                                                                   pol

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 C  G  H  K  A  I  G  T  V  L  V  G  P  T  P  V  N  I  I  G  R  N  L  L  T  Q  I  G  C  T  L  N  F  P  I  S  P  I  E  T  V  P  V  K  L  K  P  G  M
                                                                   pol

GCCCAAAAGTTAAACAATGGCCATTGACAGAAGAAAAAATAAAAGCATTAGTAGAAATTTGTACAGAAATGGAAAAGGAAGGAAAAATTTCAAAAATTGGGCCTGAGAATCCATACAATACTCCAGTATTTGCCATAAAGAAAAAG 2100
 G  P  K  V  K  Q  W  P  L  T  E  E  K  I  K  A  L  V  E  I  C  T  E  M  E  K  E  G  K  I  S  K  I  G  P  E  N  P  Y  N  T  P  V  F  A  I  K  K  K
                                                                   pol

GTACTAAATGGAGAAAATTAGTAGATTTCAGAGAACTTAATAAGAGAACTCAAGACTTCTGGGAAGTTCAATTAGGAATACCACATCCCGCAGGGTTAAAAAAGAAAAAATCAGTAACAGTACTGGATGTGGGTGATGCATATTTTT 2250
 S  T  K  W  R  K  L  V  D  F  R  E  L  N  K  R  T  Q  D  F  W  E  V  Q  L  G  I  P  H  P  A  G  L  K  K  K  K  S  V  T  V  L  D  V  G  D  A  Y  F
                                                                   pol

TTCCCTTAGATGAAGACTTCAGGAAGTATACTGCATTTACCATACCTAGTATAAACAATGAGACACCAGGGATTAGATATCAGTACAATGTGCTTCCACAGGGATGGAAAGGATCACCAGCAATATTCCAAAGTAGCATGACAAAAA 2400
 V  P  L  D  E  D  F  R  K  Y  T  A  F  T  I  P  S  I  N  N  E  T  P  G  I  R  Y  Q  Y  N  V  L  P  Q  G  W  K  G  S  P  A  I  F  Q  S  S  M  T  K
                                                                   pol

TAGAGCCTTTTAAAAAACAAAATCCAGACATAGTTATCTATCAATACATGAACGATTTGTATGTAGGATCTGACTTAGAAATAGGGCAGCATAGAACAAAAATAGAGGAGCTGAGACAACATCTGTTGAGGTGGGGACTTACCACAC 2550
 L  E  P  F  K  K  Q  N  P  D  I  V  I  Y  Q  Y  M  N  D  L  Y  V  G  S  D  L  E  I  G  Q  H  R  T  K  I  E  E  L  R  Q  H  L  L  R  W  G  L  T  T
                                                                   pol

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 D  K  K  H  Q  K  E  P  P  F  L  W  M  G  Y  E  L  H  P  D  K  W  T  V  Q  P  I  V  L  P  E  K  D  S  W  T  V  N  D  I  Q  K  L  V  G  K  L  N  T
                                                                   pol

GTCAGATTTACCCAGGGATTAAAGTAAGGCAATTATGTAAACTCCTTAGAGGAACCAAAGCACTAACAGAAGTAATACCACTAACAGAAGAAGCAGAGCTAGAACTGGCAGAAAACAGAGAGATTCTAAAAGAACCAGTACATGGAG 2850
 S  Q  I  Y  P  G  I  K  V  R  Q  L  C  K  L  L  R  G  T  K  A  L  T  E  V  I  P  L  T  E  E  A  E  L  E  L  A  E  N  R  E  I  L  K  E  P  V  H  G
                                                                   pol

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 Y  Y  D  P  S  K  D  L  I  A  E  I  Q  K  Q  G  Q  G  Q  W  T  Y  Q  I  Y  Q  E  P  F  K  N  L  K  T  G  K  Y  A  R  M  R  G  A  H  T  N  D  V  K
                                                                   pol
```

TAACAGAGGCAGTGCAAAAAATAACCACAGAAAGCATAGTAATATGGGGAAAGACTCCTAAATTTAAACTACCCATACAAAAGGAAACATGGGAAACATGGTGGACAGAGTATTGGCAAGCCACCTGGATTCCTGAGTGGGAGTTTG 3150

L T E A V Q K I T T E S I V I W G K T P K F K L P I Q K E T W E T W W T E Y W Q A T W I P E W E F

pol

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N T P P L V K L W Y Q L E K E P I V G A E T F Y V D G A A N R E T K L G K A G Y V T N K G R Q K V

pol

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P L T N T T N Q K T Q L Q A I Y L A L Q D S G L E V N I V T D S Q Y A L G I I Q A Q P D K S E S E

pol

TCAATCAAATAATAGAGCAGTTAATAAAAAAGGAAAAGGTCTATCTGGCATGGGTACCAGCACACAAAGGAATTGGAGGAAATGAACAAGTAGATAAATTAGTCAGTGCTGGAATCAGGAAAATACTATTTTTAGATGGAATAGATA 3600

V N Q I I E Q L I K K E K V Y L A W V P A H K G I G G N E Q V D K L V S A G I R K I L F L D G I D

pol

CCCAAGATGAACATTAGAATTCTGCAACAACTGCTGTTTATCCATTTTCAGAATTGGGTGTCGACATAGCAGAATAGGCGTTACTCGACAGAGGAGAGCAAGAAATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAG 3750

M E P V D P R L E P W . K H P

tat

A Q D E H .

pol

GTCAGCCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGACAGTCAGACTC 3900

S Q P K T A C T N C Y C K K C C F H C Q V C F I T K A L G I S Y G R K K R R Q R R R P P Q D S . Q T

tat

M A G R S G D S D E D L L K T V R L

rev

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Q Y S L S K Q

tat

K F L Y Q S

rev

TAGACAGGTTAATTGATAGGATAACAGAAAGAGCAGAAGACAGTGGCAATGAAAGTGAAGGGGATCAGGAAGAATTATCAGCACTTGTGGAAATGGGGCATCATGCTCCTTGGGATGTTGATGATCTGTAGTGCTGTAGAAAATTTG 4200

tat

rev

A M K V K G I R K N Y Q H L W K W G I M L L G M L M I C S A V E N L

ADA env

TGGGTCACAGTTTATTATGGGGTACCTGTGTGGAAAGAAGCAACCACCACTCTATTTTGTGCATCAGATGCTAAAGCATATGATACAGAGGTACATAATGTTTGGGCCACACATGCCTGTGTACCCACAGACCCCAACCCACAAGAAGTA 4350

tat

rev

W V T V Y Y G V P V W K E A T T T L F C A S D A K A Y D T E V H N V W A T H A C V P T D P N P Q E V

ADA env

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tat

rev

V L E N V T E N F N M W K N N M V E Q M H E D I I S L W D Q S L K P C V K L T P L C V T L N C T D L

ADA env

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tat

rev

R N V T N I N N S S E G M R G E I K N C S F N I T T S I R D K V K K D Y A L F Y R L D V V P I D N D

ADA env

AATACTAGCTATAGGTTGATAAATTGTAATACCTCAACCATTACACAGGCCTGTCCAAAGGTATCCTTTGAGCCAATTCCCATACATTATTGTACCCCGGCTGGTTTTGCGATTCTAAAGTGTAAAGACAAGAAGTTCAATGGAACAGGG 4800

tat

rev

N T S Y R L I N C N T S T I T Q A C P K V S F E P I P I H Y C T P A G F A I L K C K D K K F N G T G

ADA env

CCATGTAAAAATGTCAGCACAGTACAATGTACACATGGAATTAGGCCAGTAGTGTCAACTCAACTGCTGTTAAATGGCAGTCTAGCAGAAGAAGAGGTAGTAATTAGATCTAGTAATTTCACAGACAATGCAAAAAACATAATAGTACAG 4950

tat

rev

P C K N V S T V Q C T H G I R P V V S T Q L L L N G S L A E E E V V I R S S N F T D N A K N I I V Q

ADA env

```
AAACAATCTGTAGAAATTAATTGTACAAGACCCAACAACAATACAAGGAAAAGTATACATATAGGACCAGGAAGAGCATTTTATACAACAGGAGAAATAATAGGAGATATAAGACAAGCACATTGCAACATTAGTAGAACAAAATGG 5100
tat
rev
 K  E  S  V  E  I  N  C  T  R  P  N  N  N  T  R  K  S  I  H  I  G  P  Q  R  A  F  Y  T  T  G  E  I  I  G  D  I  R  Q  A  H  C  N  I  S  R  T  K  W
ADA env

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tat
rev
 N  T  L  N  Q  I  A  T  K  L  K  E  Q  F  G  N  N  K  T  I  V  F  N  Q  S  S  G  G  D  P  E  I  V  M  H  S  F  N  C  G  G  E  F  F  Y  C  N  S  T
ADA env

CTGTTTAATAGTACTTGGAATTTTAATGGTACTTGGAATTTAACACAATCGAATGGTACTGAAGGAAATGACACTATCACACTCCCATGTAGAATAAAACAAATTATAAATATGTGGCAGGAAGTAGGAAAAGCAATGTATGCCCCT 5400
tat
rev
 L  F  N  S  T  W  N  F  N  G  T  W  N  L  T  Q  S  N  G  T  E  G  N  D  T  I  T  L  P  C  R  I  K  Q  I  I  N  M  W  Q  E  V  G  K  A  M  Y  A  P
ADA env

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tat
rev
 I  R  G  Q  I  R  C  S  S  N  I  T  G  L  I  L  T  R  D  G  G  T  N  S  S  G  S  E  I  F  R  P  G  G  G  D  M  R  D  N  W  R  S  E  L  Y  K  Y  K
ADA env

GTAAAAATTGAACCATTAGGAGTAGCACCCACCAAGGCAAAAAGAAGAGTGGTGCAGAGAGAAAAAAGAGCAGTGGGAACGATAGGAGCTATGTTCCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCGCAGCGTCAATAACG 5700
tat
rev
 V  K  I  E  P  L  G  V  A  P  T  K  A  K  R  R  V  V  Q  R  E  K  R  A  V  G  T  I  G  A  M  F  L  G  F  L  G  A  A  G  S  T  M  G  A  A  S  I  T
ADA env
```

TGACCGTACAGGCCAGACTATTATTGTCTGGTATAGTGCAACAGCAGAACAATTTGCTGAGGGCTATTGAGGCGCAACAGCATCTGTTGCAACTCACAGTCTGGGGCATCAAGCAGCTCCAGGCAAGAGTCCTGGCTCTGGAAAGATAC 5850

tat

rev

ADA env: L T V Q A R L L L S G I V Q Q Q N N L L R A I E A Q Q H L L Q L T V W G I K Q L Q A R V L A L E R Y

TAAGGGATCAACAGCTCCTAGGGATTTGGGGTTGCTCTGGAAAACTCATCTGCACCACTGCTGTGCCTTGGAATGCTAGTTGGAGTAATAAAACTCTGGATATGATTTGGGATAACATGACCTGGATGGAGTGGGAAAGAGAAATCGAA 6000

tat

rev

ADA env: L R D Q Q L L G I W G C S G K L I C T T A V P W N A S W S N K T L D M I W D N M T W M E W E R E I E

ATTACACAGGCTTAATATACACCTTAATTGAAGAATCGCAGAACCAACAAGAAAAGAATGAACAAGACTTATTAGCATTAGATAAGTGGGCAAGTTTGTGGAATTGGTTTGACATATCAAATTGGCTGTGGTGTATAAAAATCTTCATA 6150

tat

rev

ADA env: N Y T G L I Y T L I E E S Q N Q Q E K N E Q D L L A L D K W A S L W N W F D I S N W L W C I K I F I

TGATAGTAGGAGGCTTGATAGGTTTAAGAATAGTTTTTACTGTACTTTCTATAGTAAATAGAGTTAGGCAGGGATACTCACCATTGTCATTTCAGACCCACCTCCCAGCCCCGAGGGGACCCGACAGGCCCGAAGGAATCGAAGAAGAA 6300

tat: P T S Q P R G D P T G P K E S K K K

rev: N P P P S P E G T R Q A R R N R R R

ADA env: M I V G G L I G L R I V F T V L S I V N R V R Q G Y S P L S F Q T H L P A P R G P D R P E G I E E E

GTGGAGACAGAGACAGAGACAGATCCGTCCATTAGTGGATGGATCCTTAGCACTTATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCAGCTACCACCGCTTGAGAGACTTACTCTTGATTGTAACGAGGATTGTGGAACTTCTGGGA 6450

tat: V E T E T E T D P C D .

rev: W R Q R Q R Q I R A I S G W I L S T Y L G R S A E P V P L Q L P P L E R L T L D C N E D C G T S G

ADA env: G G D R D R D R S V R L V D G S L A L I W D D L R S L C L F S Y H R L R D L L L I V T R I V E L L G

GCAGGGGGTGGGAAGCCCTCAAATATTGGTGGAATCTCCTACAGTATTGGAGTCAGGAGCTAAAGAATAGTGCTGTTAGCTTGCTCAATGCCACAGCTATAGCAGTAGCTGAGGGGACAGATAGGGTTATAGAAGTAGTACAAGGAGCT 6600

rev: Q G V Q S P Q I L V E S P T V L E S G A K E .

ADA env: R R G W E A L K Y W W N L L Q Y W S Q E L K N S A V S L L N A T A I A V A E G T D R V I E V V Q G A

Site and Sequence

AGAGCTATTCGCCACATACCTAGAACAATAACACAGGCTTGGAAAGGATTTTGCTATAAGATGGGTGGCTAGCCCCGGGTGATAAACGGACCGCGCAATCCCTAGGCTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTC 6750

pGA2

R A I R H I P R R I R Q G L E R I L L .

ADA env

CGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAA 6900

pGA2

CAGGCATGCTGGGGATGCGGTGGGCTCTATATAAAAAACGCCCGGCGGCAACCGAGCGTTCTGAACGCTAGAGTCGACAAATTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGT 7050

pGA2

GCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCTGCCACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCG 7200

pGA2

AGCAGGCATCGCCATGGGTCACGACGAGATCCTCGCCGTCGGGCATGCTCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTG 7350

pGA2

CTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCA 7500

pGA2

GCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCTTGCAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAACCGGGC 7650

pGA2

CCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCATGCGAAACGATCCTCATCCTG 7800

pGA2

CTTGATCAGATCTTGATCCCCTGCGCCATCAGATCCTTGGCGGCGAGAAAGCCATCCAGTTTACTTTGCAGGGCTTCCCAACCTTACCAGAGGGCGCCCCAGCTGGCAATTCCGGTTCGCTTGCTGTCCATAAAACCGCCCAGTCTA 7950

pGA2

ATCGCCATGTAAGCCCACTGCAAGCTACCTGCTTTCTCTTTGCGCTTGCGTTTTCCCTTGTCCAGATAGCCCAGTAGCTGACATTCATCCGGGGTCAGCACCGTTTCTGCGGACTGGCTTTCTACGTGAAAAGGATCTAGGTGAAGA 8100

pGA2

TTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTAC 8250

pGA2

CGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACC 8400

pGA2

TCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGA 8550

pGA2

CTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTT 8700

pGA2

TAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGGCTTTTGCTGGCCTTTTGCTCACATGTTGTCGACCG 8850

pGA2

CAATATTGGCTATTGGCCATTGCATACGTTGTATCTATATCATAATATGTACATTTATATTGGCTCATGTCCAATATGACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCC 9000

pGA2

ATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTCGTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTT 9150

pGA2

CGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTCCGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTACGGGACTTTCCTACTTGGCAGTACATCTACGTATTA 9300

pGA2

TCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACACCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGT 9450

pGA2

GTAATAACCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGC 9545

pGA2

Enzymes : 22 of 500 enzymes (Filtered)

Settings : Circular, Subrange Context, Certain Sites Only, Standard Genetic Code

```
Cla I                                                                                                                                        Cla I
ATCGATGCAGGACTCGGCTTGCTGAAGCGCGCACGGCAAGAGGCGAGGGGCGGCGACTGGTGAGTACGCCAAAAATTTTGACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGATCGA 150
                                                                                                         M G A R A S V L S G G E L D R
                                                                                                         gag

  Xmn I
TGGGAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAACATATAGTATGGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACATCAGAAGGCTGTAGACAAATACTGGGACAGCTACAA 300
W E K I R L R P G G K K K Y K L K H I V W A S R E L E R F A V N P G L L E T S E G C R Q I L G Q L Q
gag

                                                                                                 Hind III
CCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATATAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAAGCA 450
P S L Q T G S E E L R S L Y N T V A T L Y C V H Q R I E I K D T K E A L D K I E E E Q N K S K K K A
gag

                                                                                                                                                Xmn I
CAGCAAGCAGCAGCTGACACAGGACACAGCAGTCAGGTCAGCCAAAATTACCCTATAGTGCAGAACATCCAGGGGCAAATGGTACATCAGGCCATATCACCTAGAACTTTAAATGCATGGGTAAAAGTAGTAGAAGAGAAGGCTTTCAGC 600
Q Q A A A D T G H S S Q V S Q N Y P I V Q N I Q G Q M V H Q A I S P R T L N A W V K V V E E K A F S
gag

                                                                                                                                     Pst I
CCAGAAGTAATACCCATGTTTTCAGCATTATCAGAAGGAGCCACCCCACAAGATTTAAACACCATGCTAAACACAGTGGGGGGACATCAAGCAGCCATGCAAATGTTAAAAGAGACCATCAATGAGGAAGCTGCAGAATGGGATAGAGTA 750
P E V I P M F S A L S E G A T P Q D L N T M L N T V G G H Q A A M Q M L K E T I N E E A A E W D R V
gag

          Sph I
CATCCAGTGCATGCAGGGCCTATTGCACCAGGCCAGATGAGAGAACCAAGGGGAAGTGACATAGCAGGAACTACTAGTACCCTTCAGGAACAAATAGGATGGATGACAAATAATCCACCTATCCCAGTAGGAGAAATTTATAAAAGATGG 900
H P V H A G P I A P G Q M R E P R G S D I A G T T S T L Q E Q I G W M T N N P P I P V G E I Y K R W
gag

                                                                                                   Age I                         Hind III
ATAATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTACCAGCATTCTGGACATAAGACAAGGACCAAAAGAACCTTTTAGAGACTATGTAGACCGGTTCTATAAAACTCTAAGAGCCGAGCAAGCTTCACAGGAGGTAAAAAAT 1050
I I L G L N K I V R M Y S P T S I L D I R Q G P K E P F R D Y V D R F Y K T L R A E Q A S Q E V K N
gag

TGGATGACAGAAACCTTGTTGGTCCAAAATGCGAACCCAGATTGTAAGACTATTTTAAAAGCATTGGGACCAGCGGCTACACTAGAAGAAATGATGACAGCATGTCAGGGAGTAGGAGGACCCGGCCATAAGGCAAGAGTTTTGGCTGAA 1200
W M T E T L L V Q N A N P D C K T I L K A L G P A A T L E E M M T A C Q G V G G P G H K A R V L A E
gag
```

Apa I
Avr II

GCAATGAGCCAACTAACAAATACAGCTACCATAATGATGCAGAGAGGCAATTTTAGGAACCAAAGAAAGATGGTTAAGAGCTTCAATAGCGGCAAAGAAGGGCACACAGCCAGAAATTGCAGGGCCCCTAGGAAAAAGGGCAGCTGGAAA 1350
A M S Q V T N T A T I M M Q R G N F R N Q R K N Y K S F N S G K E G H T A R N C R A P R K K G S V K
gag

Bgl II

AGCGGAAAGGAAGGACACCAAATGAAAGATTGTACTGAGAGACAGGCTAATTTTTTAGGGAAGATCTGGCCTTCCTACAAGGGAAGGCCAGGGAATTTTCTTCAGAGCAGACCAGAGCCAACAGCCCCACCATTTCTTCAGAGCAGACCA 1500
S G K E G H Q M K D C T E R Q A N F L G K I W P S Y K G R P G N F L Q S R P E P T A P P F L Q S R P
gag
F F R E D L A F L Q G K A R E F S S E Q T R A N S P T I S S E Q T
pol (deleted integrase)

GAGCCAACAGCCCCACCAGAAGAGAGCTTCAGGTCTGGGGTAGAGACAACAACTCCCCCTCAGAAGCAGGAGCCGATAGACAAGGAACTGTATCCTTTAACTTCCCTCAGATCACTCTTTGGCAACGACCCCTCGTCACAATAAAGATAG 1650
E P T A P P E E S F R S G V E T T T P P Q K Q E P I D K E L Y P L T S L R S L F G N D P S S Q .
gag
R A N S P T R R E L Q V W G R D N N S P S E A G A D R Q G T V S F N F P Q I T L W Q R P L V T I K I
pol (deleted integrase)

GGGGGCAACTAAAGGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGAGTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTTATCAAAGTAAGACAGTATGATCAGATACTCATAGAAA 1800
G G Q L K E A L L D T G A D D T V L E E M S L P G R W K P K M I G G I G G F I K V R Q Y D Q I L I E
pol (deleted integrase)

TCTGTGGACATAAAGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGGAAGAAATCTGTTGACTCAGATTGGTTGCACTTTAAATTTTCCCATTAGCCCTATTGAGACTGTACCAGTAAAATTAAAGCCAGGAATGG 1950
I C G H K A I G T V L V G P T P V N I I G R N L L T Q I G C T L N F P I S P I E T V P V K L K P G M
pol (deleted integrase)

ATGGCCCAAAAGTTAAACAATGGCCATTGACAGAAGAAAAAATAAAAGCATTAGTAGAAATTTGTACAGAAATGGAAAAGGAAGGGAAAATTTCAAAAATTGGGCCTGAGAATCCATACAATACTCCAGTATTTGCCATAAAGAAAAAAG 2100
D G P K V K Q W P L T E E K I K A L V E I C T E M E K E G K I S K I G P E N P Y N T P V F A I K K K
pol (deleted integrase)

ACAGTACTAAATGGAGAAAATTAGTAGATTTCAGAGAACTTAATAAGAGAACTCAAGACTTCTGGGAAGTTCAATTAGGAATACCACATCCCGCAGGGTTAAAAAAGAAAAAATCAGTAACAGTACTGGATGTGGGTGATGCATATTTTT 2250
D S T K W R K L V D F R E L N K R T Q D F W E V Q L G I P H P A G L K K K K S V T V L D V G D A Y F
pol (deleted integrase)

EcoR V

CAGTTCCCTTAGATGAAGACTTCAGGAAGTATACTGCATTTACCATACCTAGTATAAACAATGAGACACCAGGGATTAGATATCAGTACAATGTGCTTCCACAGGGATGGAAAGGATCACCAGCAATATTCCAAAGTAGCATGACAAAAA 2400
S V P L D E D F R K Y T A F T I P S I N N E T P G I R Y Q Y N V L P Q G W K G S P A I F Q S S M T K
pol (deleted integrase)

TCTTAGAGCCTTTTAAAAAACAAAATCCAGACATAGTTATCTATCAATACATGAACGATTTGTATGTAGGATCTGACTTAGAAATAGGGCAGCATAGAACAAAAATAGAGGAGCTGAGACAACATCTGTTGAGGTGGGGACTTACCACAC 2550

I L E P F K K Q N P D I V I Y Q Y M N D L Y V G S D L E I G Q H R T K I E E L R Q H L L R W G L T T

pol (deleted integrase)

CAGACAAAAAACATCAGAAAGAACCTCCATTCCTTTGGATGGGTTATGAACTCCATCCTGATAAATGGACAGTACAGCCTATAGTGCTGCCAGAAAAAGACAGCTGGACTGTCAATGACATACAGAAGTTAGTGGGGAAATTGAATACCG 2700

P D K K H Q K E P P F L W M G Y E L H P D K W T V Q P I V L P E K D S W T V N D I Q K L V G K L N T

pol (deleted integrase)

CAAGTCAGATTTACCCAGGGATTAAAGTAAGGCAATTATGTAAACTCCTTAGAGGAACCAAAGCACTAACAGAAGTAATACCACTAACAGAAGAAGCAGAGCTAGAACTGGCAGAAAACAGAGAGATTCTAAAAGAACCAGTACATGGAG 2850

A S Q I Y P G I K V R Q L C K L L R G T K A L T E V I P L T E E A E L E L A E N R E I L K E P V H G

pol (deleted integrase)

TGTATTATGACCCATCAAAAGACTTAATAGCAGAAATACAGAAGCAGGGGCAAGGCCAATGGACATATCAAATTTATCAAGAGCCATTTAAAAATCTGAAAACAGGAAAATATGCAAGAATGAGGGGTGCCCACACTAATGATGTAAAAC 3000

V Y Y D P S K D L I A E I Q K Q G Q G Q W T Y Q I Y Q E P F K N L K T G K Y A R M R G A H T N D V K

pol (deleted integrase)

AATTAACAGAGGCAGTGCAAAAAATAACCACAGAAAGCATAGTAATATGGGGAAAGACTCCTAAATTTAAACTACCCATACAAAAGGAAACATGGGAAACATGGTGGACAGAGTATTGGCAAGCCACCTGGATTCCTGAGTGGGAGTTTG 3150

Q L T E A V Q K I T T E S I V I W G K T P K F K L P I Q K E T W E T W W T E Y W Q A T W I P E W E F

pol (deleted integrase)

Kpn I

TTAATACCCCTCCTTTAGTGAAATTATGGTACCAGTTAGAGAAAGAACCCATAGTAGGAGCAGAAACCTTCTATGTAGATGGGGCAGCTAACAGGGAGACTAAATTAGGAAAAGCAGGATATGTTACTAACAAAGGAAGACAAAAGGTTG 3300

V N T P P L V K L W Y Q L E K E P I V G A E T F Y V D G A A N R E T K L G K A G Y V T N K G R Q K V

pol (deleted integrase)

TCCCCCTAACTAACACAACAAATCAGAAAACTCAGTTACAAGCAATTTATCTAGCTTTGCAGGATTCAGGATTAGAAGTAAACATAGTAACAGACTCACAATATGCATTAGGAATCATTCAAGCACAACCAGATAAAAGTGAATCAGAGT 3450

V P L T N T T N Q K T Q L Q A I Y L A L Q D S G L E V N I V T D S Q Y A L G I I Q A Q P D K S E S E

pol (deleted integrase)

Kpn I

TAGTCAATCAAATAATAGAGCAGTTAATAAAAAAGGAAAAGGTCTATCTGGCATGGGTACCAGCACACAAAGGAATTGGAGGAAATGAACAAGTAGATAAATTAGTCAGTGCTGGAATCAGGAAAATACTATTTTTAGATGGAATAGATA 3600

L V N Q I I E Q L I K K E K V Y L A W V P A H K G I G G N E Q V D K L V S A G I R K I L F L D G I D

pol (deleted integrase)

EcoR I

Sal I

AGGCCCAAGATGAACATTAGAATTCTGCAACAACTGCTGTTTATCCATTTTCAGAATTGGGTGTCGACATAGCAGAATAGGCGTTACTCGACAGAGGAGAGCAAGAAATGGAGCCAGTAGATCCTAGACTAGAGCCCTGGAAGCATCCAG 3750

M E P V D P R L E P W K H P

tat

K A Q D E H .

pol (deleted integrase)

```
          Mun I
GAAGTCAGCCTAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTTTGTTTCATAACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGACCTCCTCAAGGCAGTCAGACTC 3900
G S Q P K T A C T N C Y C K K C C F H C Q V C F I T K A L G I S Y G R K K R R Q R R R P P Q G S Q T
                        tat
                                                         M A G R S G D S D E D L L K A V R L
                                                                    rev

ATCAAGTTTCTCTATCAAAGCAGTAAGTAGTACATGTAATGCAACCTATACAAATAGCAATAGTAGCATTAGTAGTAGCAATAATAATAGCAATAGTTGTGTGGTCCATAGTAATCATAGAATATAGGAAAATATTAAGACAAAGAAAA 4050
H Q V S L S K Q
                        tat
I K F L Y Q S
                        rev
           M Q P I Q I A I V A L V V A I I I A I V V W S I V I I E Y R K I L R Q R K
                        vpu

TAGACAGGTTAATTGATAGACTAATAGAAAGAGCAGAAGACAGTGGCAATGAGAGTGAAGGAGAAATATCAGCACTTGTGGAGATGGGGGTGGAGATGGGGCACCATGCTCCTTGGGATGTTGATGATCTGTAGTGCTACAGAAAAATTG 4200
                        tat
                        rev
I D R L I D R L I E R A E D S G N E S E G E I S A L V E M G V E M G H H A P W D V D D L .
                        vpu
              M R V K E K Y Q H L W R W G W R W G T M L L G M L M I C S A T E K L
                        truncated env

          Kpn I                                 Nde I
TGGGTCACAGTCTATTATGGGGTACCTGTGTGGAAGGAAGCAACCACCACTCTATTTTGTGCATCAGATGCTAAAGCATATGATACAGAGGTACATAATGTTTGGGCCACACATGCCTGTGTACCCACAGACCCCAACCCACAAGAAGTA 4350
                        tat
                        rev
W V T V Y Y G V P V W K E A T T T L F C A S D A K A Y D T E V H N V W A T H A C V P T D P N P Q E V
                        truncated env

GTATTGGTAAATGTGACAGAAAATTTTAACATGTGGAAAAATGACATGGTAGAACAGATGCATGAGGATATAATCAGTTTATGGGATCAAAGCCTAAAGCCATGTGTAAAATTAACCCCACTCTGTGTTAGTTTAAAGTGCACTGATTTG 4600
                        tat
                        rev
V L V N V T E N F N M W K N D M V E Q M H E D I I S L W D Q S L K P C V K L T P L C V S L K C T D L
                        truncated env
```

```
AAGAATCATACTAATACCAATAGTAGTAGCGGGAGAATGATAATGGAGAAAGGAGAGATAAAAAACTGCTCTTTCAATATCAGCACAAGCATAAGAGGTAAGGTGCAGAAAGAATATGCATTTTTTTATAAACTTGATATAATACCAATA 4650
------------------------------------------------------------------ tat ------------------------------------------------------------------
------------------------------------------------------------------ rev ------------------------------------------------------------------
 K  N  D  T  N  T  N  S  S  S  G  R  M  I  M  E  K  G  E  I  K  N  C  S  F  N  I  S  T  S  I  R  G  K  V  Q  K  E  Y  A  F  F  Y  K  L  D  I  I  P  I
------------------------------------------------------------- truncated env -------------------------------------------------------------

CATAATGATACTACCAGCTATACGTTGACAAGTTGTAACACCTCAGTCATTACACAGGCCTGTCCAAAGGTATCCTTTGAGCCAATTCCCATACATTATTGTGCCCCGGCTGGTTTTGCGATTCTAAAATGTAATAATAAGACGTTCAAT 4800
------------------------------------------------------------------ tat ------------------------------------------------------------------
------------------------------------------------------------------ rev ------------------------------------------------------------------
 D  N  D  T  T  S  Y  T  L  T  S  C  N  T  S  V  I  T  Q  A  C  P  K  V  S  F  E  P  I  P  I  H  Y  C  A  P  A  G  F  A  I  L  K  C  N  N  K  T  F  N
------------------------------------------------------------- truncated env -------------------------------------------------------------

                                                                                                                  Bgl II                                  Mun I
GGAACAGGACCATGTACAAATGTCAGCACAGTACAATGTACACATGGAATTAGGCCAGTAGTATCAACTCAACTGCTGTTAAATGGCAGTCTGGCAGAAGAAGAGGTAGTAATTAGATCTTCAGACCTGGAGGAGGAGATATGACGGACA 4950
------------------------------------------------------------------ tat ------------------------------------------------------------------
------------------------------------------------------------------ rev ------------------------------------------------------------------
 G  T  G  P  C  T  N  V  S  T  V  Q  C  T  H  G  I  R  P  V  V  S  T  Q  L  L  L  N  G  S  L  A  E  E  E  V  V  I  R  S  S  D  L  E  E  E  I  .
------------------------------------------------------------- truncated env ------------------------------------------------------

ATTGGAGAAGTGAATTATATAAATATAAAGTAGTAAAAATTGAACCATTAGGAGTAGCACCCACCAAGGCAAAGAGAAGAGTGGTGCAGAGAGAAAAAAGAGCAGTGGGAATAGGAGCTTTGTTCCTTGGGTTCTTGGGAGCAGCAGGAA 5100
------------------------------------------------------------------ tat ------------------------------------------------------------------
------------------------------------------------------------------ rev ------------------------------------------------------------------

GCACTATGGGCGCAGCGTCAATGACGCTGACGGTACAGGCCAGACAATTATTGTCTGGTATAGTGCAGCAGCAGAACAATTTGCTGAGGGCTATTGAGGCGCAACAGCATCTGTTGCAACTCACAGTCTGGGGCATCAAGCAGCTCCAGG 5250
------------------------------------------------------------------ tat ------------------------------------------------------------------
------------------------------------------------------------------ rev ------------------------------------------------------------------

CAAGAATCCTGGCTGTGGAAAGATACCTAAAGGATCAACAGCTCCTGGGGATTTGGGGTTGCTCTGGAAAACTCATTTGCACCACTGCTGTGCCTTGGAATGCTAGTTGGAGTAATAAATCTCTGGAACAGATTTGGAATAACATGACCT 5400
------------------------------------------------------------------ tat ------------------------------------------------------------------
------------------------------------------------------------------ rev ------------------------------------------------------------------
```

Hind III

GCATCCAGTGGCACAGAGAAATTAACAATTACACAAGCTTAATACACTCCTTAATTGAAGAATCGCAAAACCAGCAAGAAAAGAATGAACAAGAATTATTGGAATTAGATAAATGGGCAACTTTGTGGAATTGGTTTAACATAACAAATT 5550

tat

rev

GGCTGTGGTATATAAAATTATTCATAATGATAGTAGGAGGCTTGGTAGGTTTAAGAATAGTTTTTGCTGTACTTTCTGTAGTGAATAGAGTTAGGCAGGGATATTCACCATTATCGTTTCAGACCCACCTCCCAATCCCGAGGGGACCCG 5700

tat P T S Q S R G D P

rev N P P P K P E G T R

BamH I

ACAGGCCCGAAGGAATAGAAGAAGAAGGTGGAGAGAGAGACAGAGACAGATCCATTCGATTAGTGAACGGATCCTTAGCACTTATCTGGGACGATCTGCGGAGCCTGTGCCTCTTCAGCTACCACCGCTTGAGAGACTTACTCTTGATTG 5850

T G P K E .

tat

Q A R R N R R R R W R E R Q R Q I H S I S E R I L S T Y L G R S A E P V P L Q L P P L E R L T L D C

rev

TAACGAGGATTGTGGAACTTCTGGGACGCAGGGGGTGGGAAGCCCTCAAATATTGGTGGAATCTCCTACAGTATTGGAGTCAGGAGCTAAAGAATAGTGCTGTTAGCTTGCTCAATGCCACAGCTATAGCAGTAGCTGAGGGGACAGATA 6000

N E D C G T S G T Q G V G S P Q I L V E S P T V L E S G A K E .

rev

Nhe I

Avr II

GGGTTATAGAAGTAGTACAAGGAGCTTATAGAGCTATTCGCCACATACCTAGAAGAATAAGACAGGGCTTGGAAAGGATTTTGCTATAAGATGGGTGGCTAGCCCCGGGTGATAAACGGACCGCGCAATCCCTAGGCTGTGCCTTCTAGT 6150

pGA1

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pGA1

Sph I

Sal I

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pGA1

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pGA1

Nco I, Sph I

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pGA1

CAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACA 6900

pGA1

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pGA1

Kas I

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pGA1

Bgl II, Kas I

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pGA1

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pGA1

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pGA1

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pGA1

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pGA1

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pGA1

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pGA1

Sal I

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pGA1

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pGA1

Nde I

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pGA1

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pGA1

Sph I

CTCCATAGAAGACACCGGGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATTCCCCGTGCCAAGAGTGACGTAAGTACCGCCTATAGACTCTATAGGCACACCCCTTTGGCTCTTATGCATGCTATACTGTTTTTG 9150

pGA1

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pGA1

Nco I

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pGA1

Pst I

TTCTGCAGTCACCGTCCA 9918

pGA1

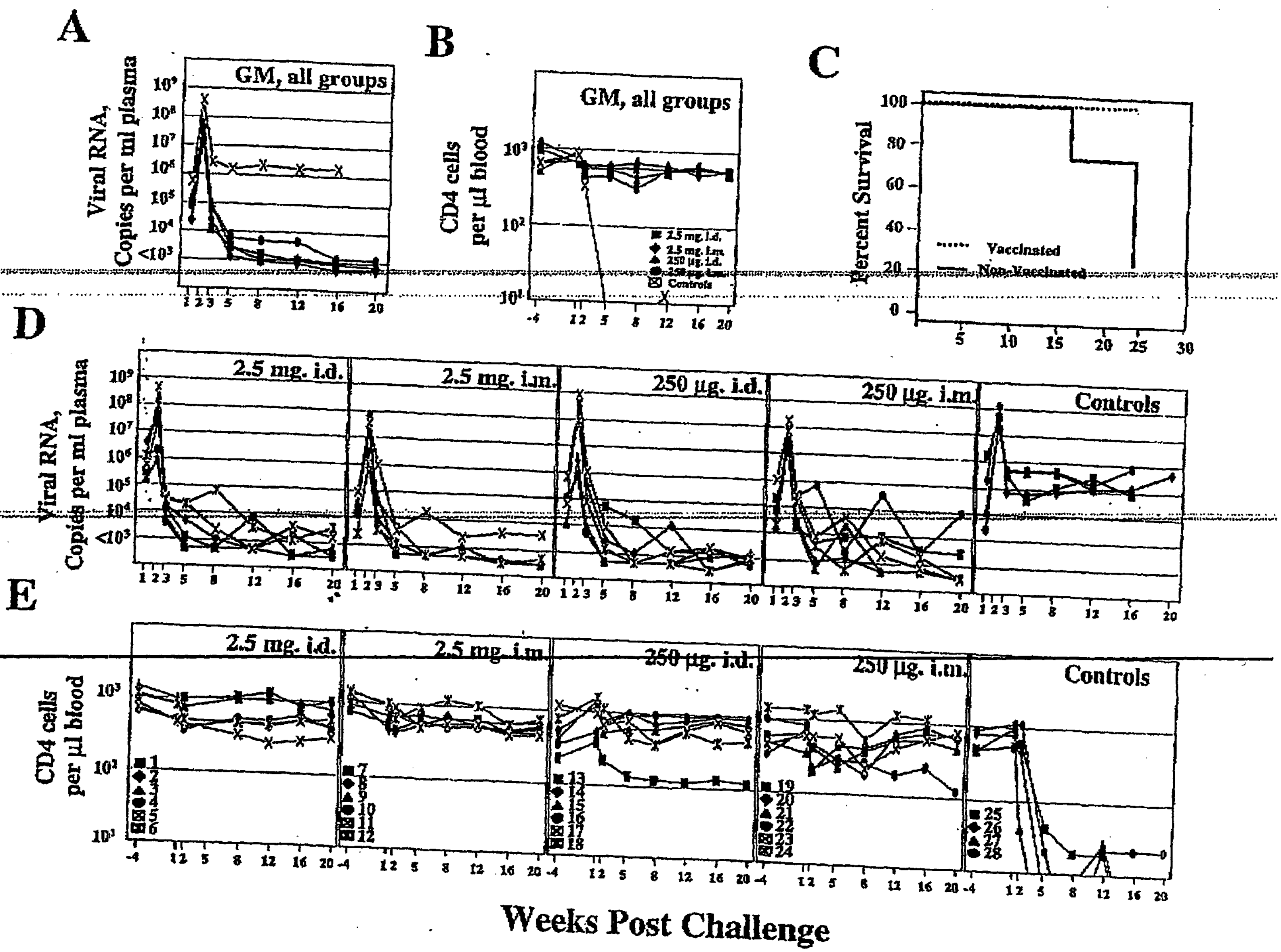

A
GM, all groups
Viral RNA, Copies per ml plasma
B
GM, all groups
CD4 cells per µl blood
2.5 mg. i.d.
2.5 mg. i.m.
250 µg. i.d.
Controls
C
Percent Survival
Vaccinated
Non-Vaccinated
D
2.5 mg. i.d.
2.5 mg. i.m.
250 µg. i.d.
250 µg. i.m.
Controls
E
Weeks Post Challenge

FIGURE 19

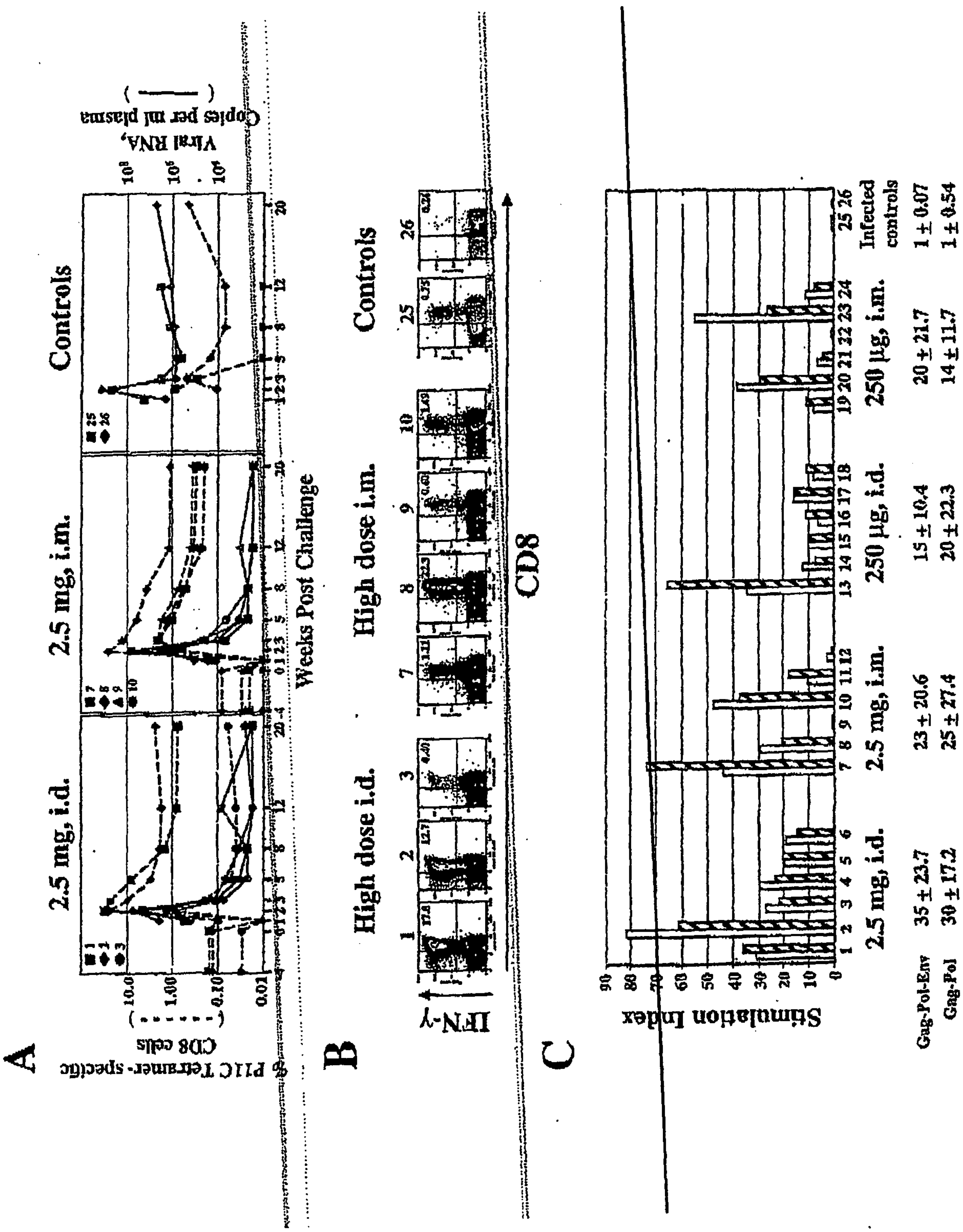

A
B
C
2.5 mg, i.d.
2.5 mg, i.m.
Controls
% P11C Tetramer - specific CD8 cells
Viral RNA, Copies per ml plasma
Weeks Post Challenge
High dose i.d.
High dose i.m.
Controls
IFN-γ
CD8
Stimulation Index
2.5 mg, i.d.
2.5 mg, i.m.
250 μg, i.d.
250 μg, i.m.
Infected controls
Gag-Pol-Env
Gag-Pol
35 ± 23.7
30 ± 17.2
23 ± 20.6
25 ± 27.4
15 ± 10.4
20 ± 22.3
20 ± 21.7
14 ± 11.7
1 ± 0.07
1 ± 0.54

FIGURE 20

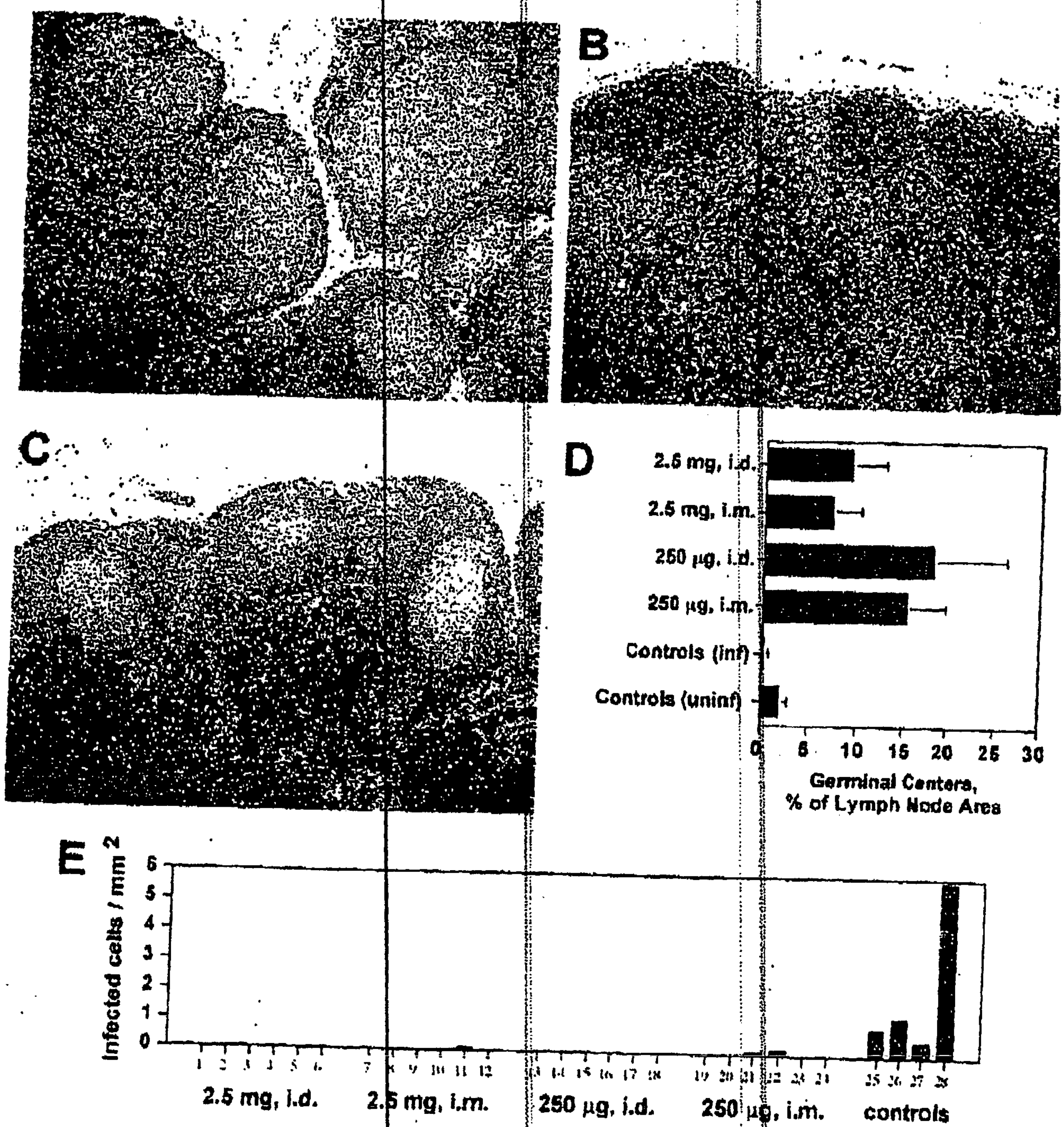

B
C
D
2.5 mg, i.d.
2.5 mg, i.m.
250 µg, i.d.
250 µg, i.m.
Controls (inf)
Controls (uninf)
0 5 10 15 20 25 30
Germinal Centers,
% of Lymph Node Area
E
Infected cells / mm 2
0 1 2 3 4 5 6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
2.5 mg, i.d.
2.5 mg, i.m.
250 µg, i.d.
250 µg, i.m.
controls

FIGURE 21

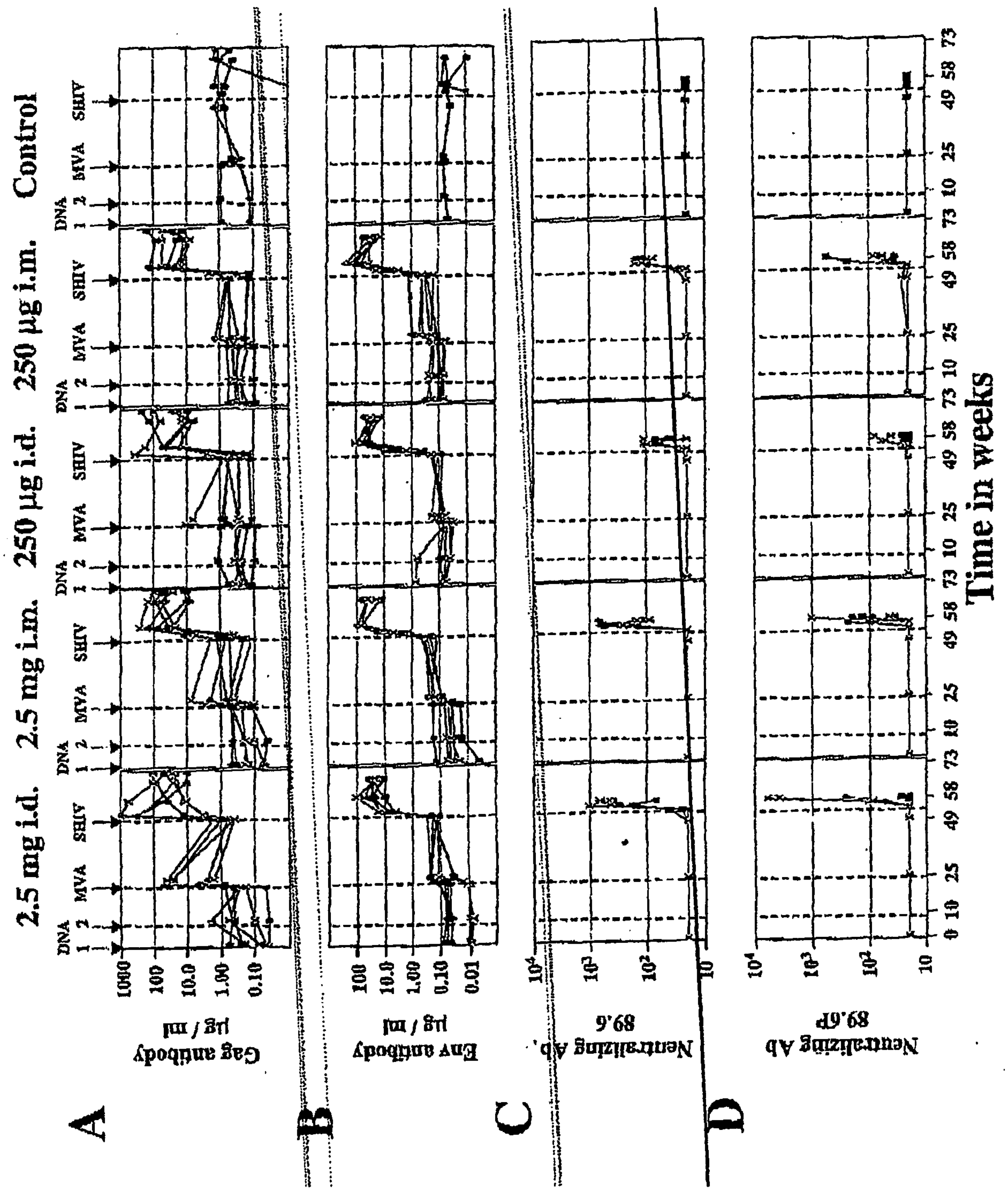

2.5 mg i.d.
2.5 mg i.m.
250 µg i.d.
250 µg i.m.
Control
DNA 1 2
MVA
SHIV
A
Gag antibody µg / ml
B
Env antibody µg / ml
C
Neutralizing Ab. 89.6
D
Neutralizing Ab 89.6P
Time in weeks

FIGURE 22

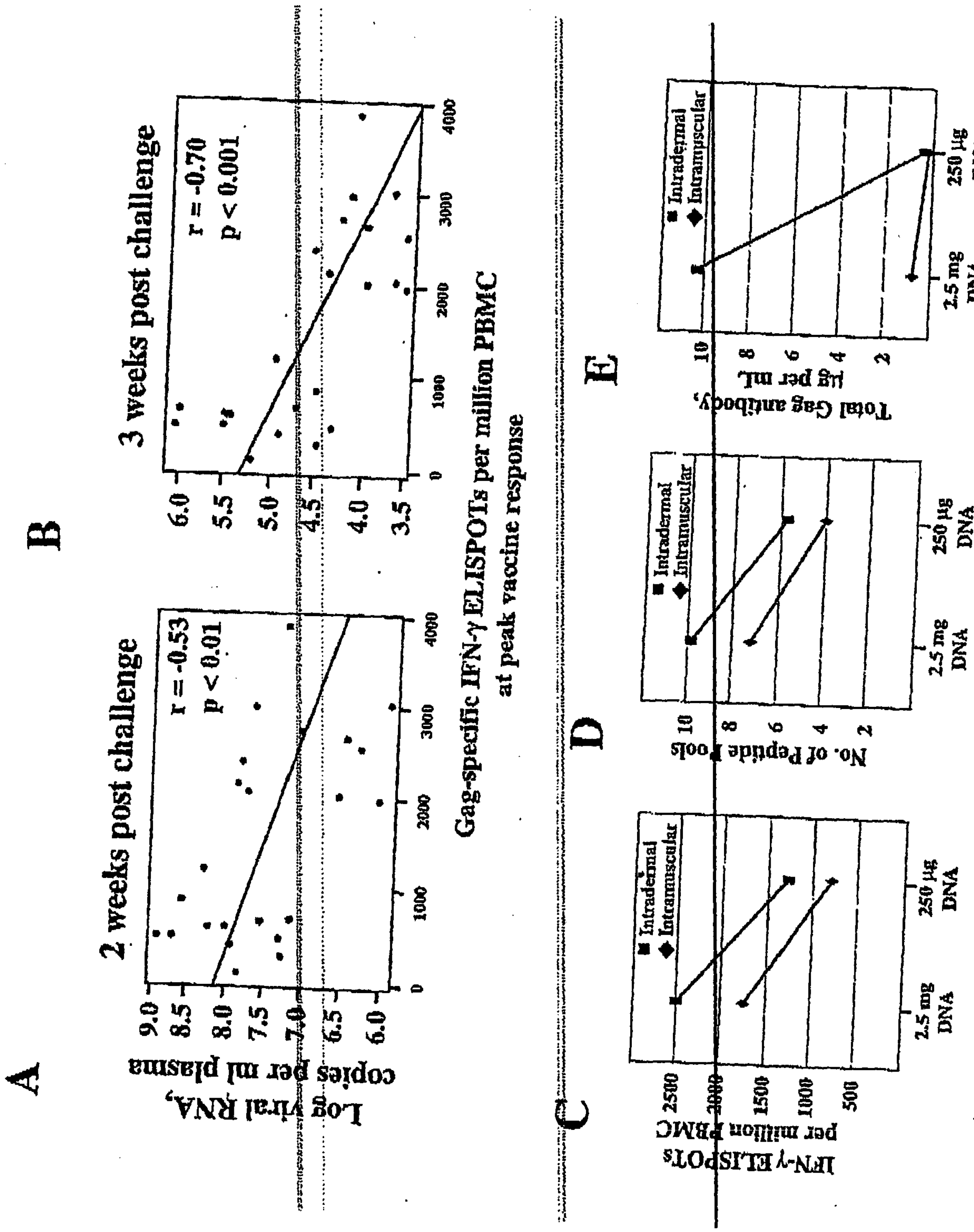
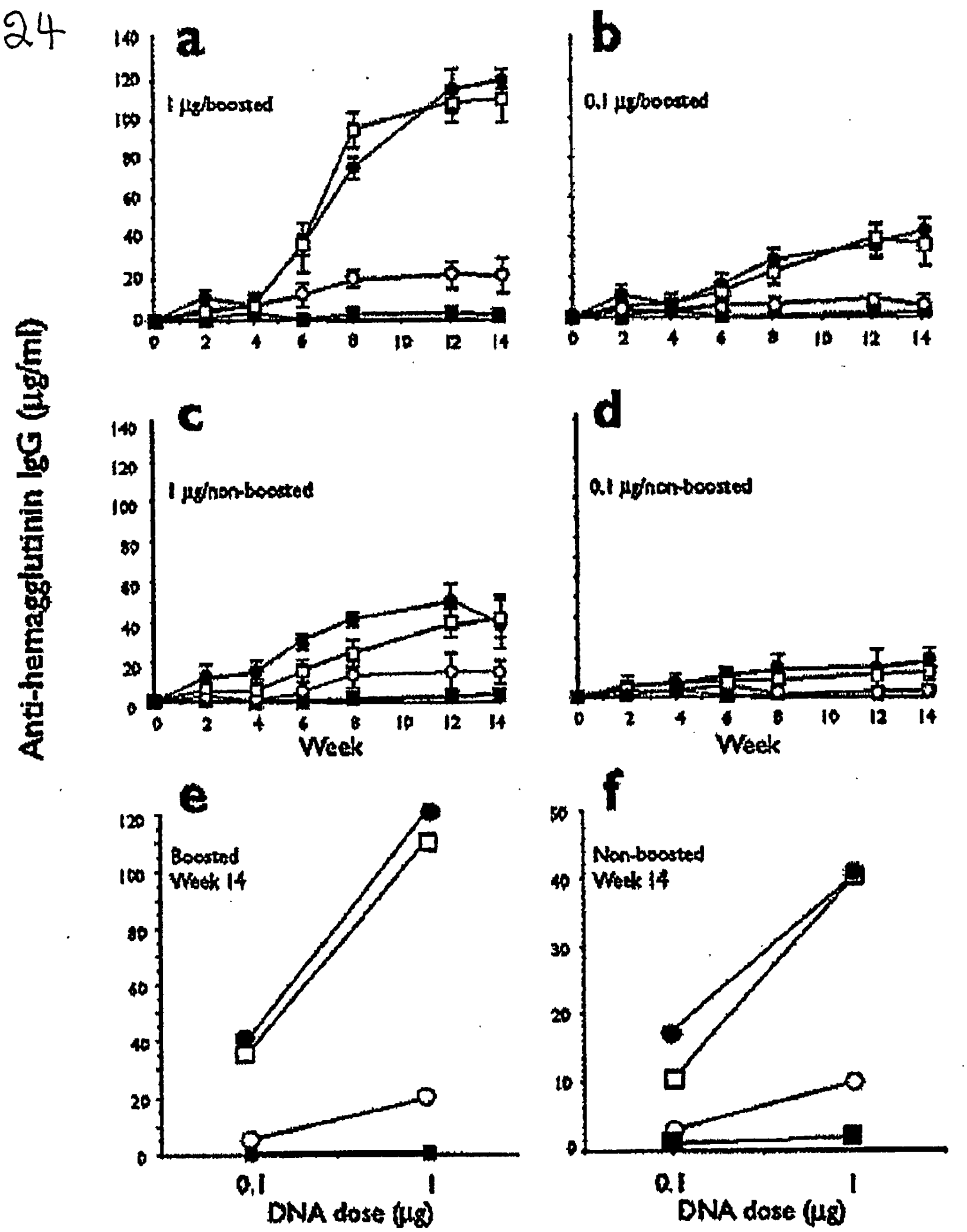

A
B
C
D
E
2 weeks post challenge
3 weeks post challenge
r = -0.53
p < 0.01
r = -0.70
p < 0.001
Log viral RNA, copies per ml plasma
Gag-specific IFN-γ ELISPOTs per million PBMC at peak vaccine response
IFN-γ ELISPOTs per million PBMC
No. of Peptide Pools
Total Gag antibody, µg per mL
Intradermal
Intramuscular
2.5 mg DNA
250 µg DNA

FIGURE 23

Fig. 24



~~Figure 23~~. Anti-HA IgG raised by gene gun inoculation of DNAs expressing HA proteins. Mice were immunized with different doses of vaccine plasmid. Half of the mice were primed at day 0 and boosted at week 4 (a,b) and half were given a single vaccination at day 0 (c,d). A ratio of the dose of DNA to specific IgG concentrations was obtained at week 14 (e,f). Sera were obtained from mice with vector (filled squares), sHA (open circles), tmHA (open squares) or sHA-3C3d (filled circles). Sera collected at the indicated times from each group were pooled for determination of specific IgG levels by ELISA. Data are represented as the average of three individual assays. Preimmune sera from mice had no detectable specific IgG.

Figure 24

Fig. 25

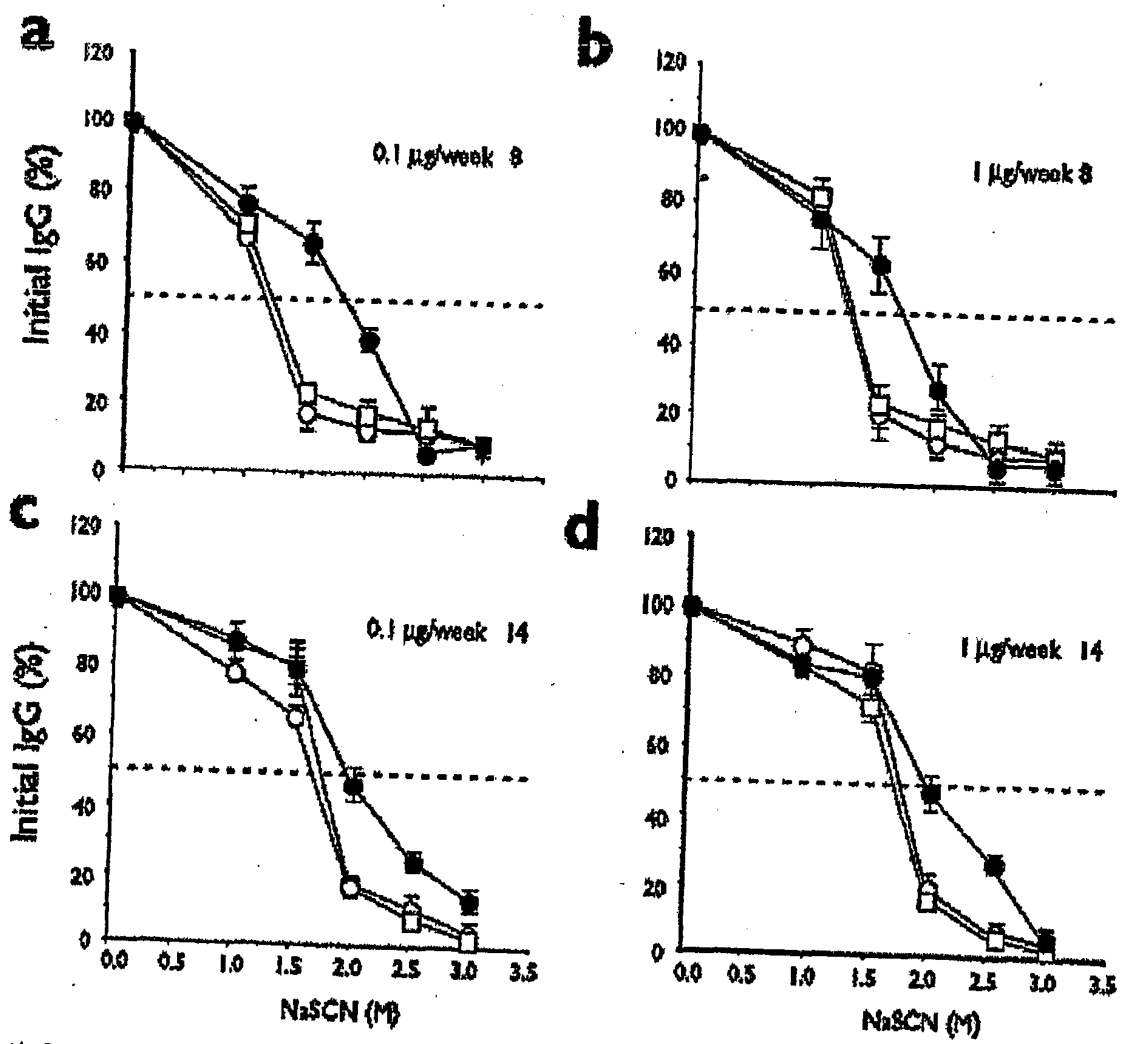
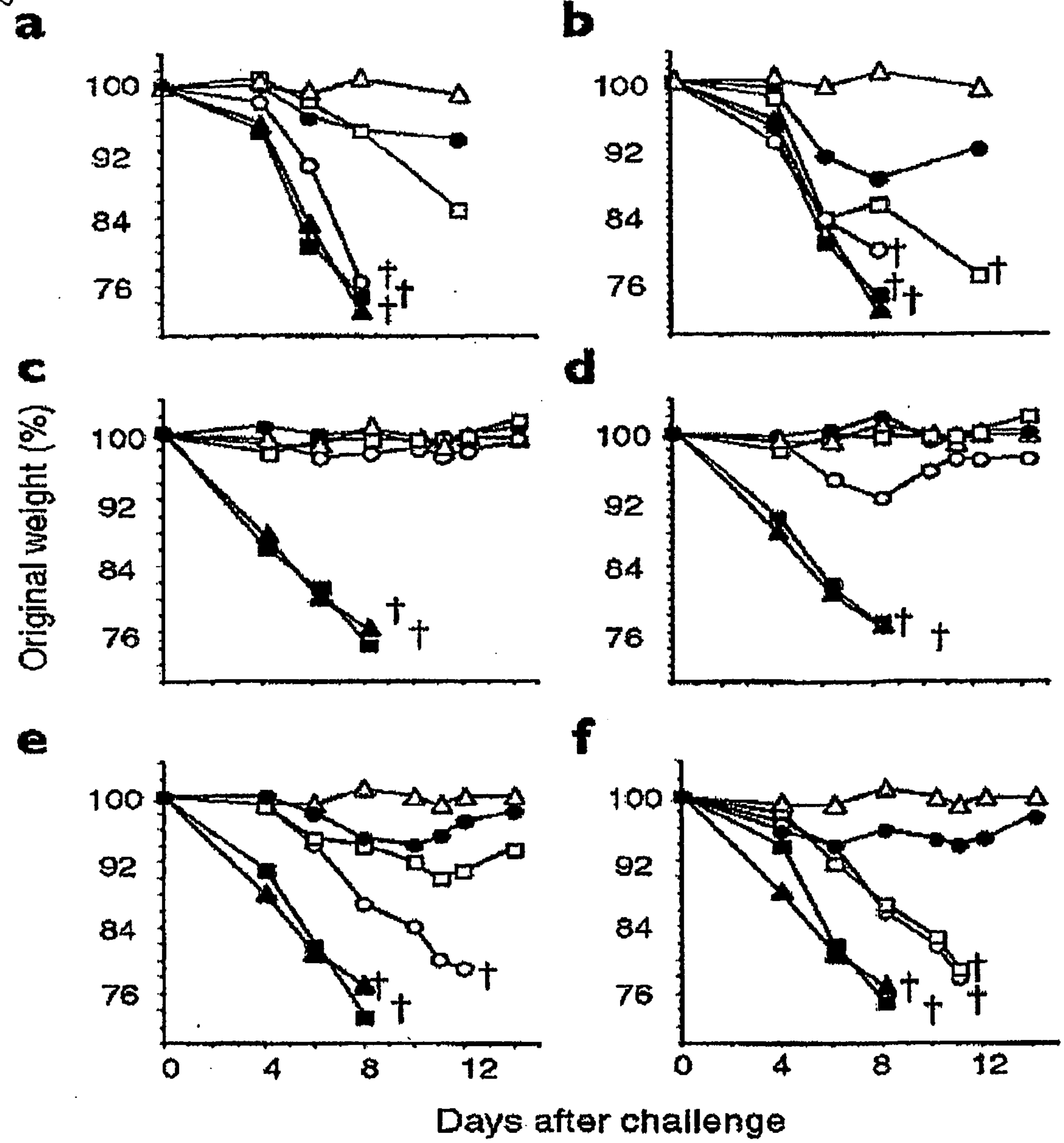


Figure 4 Avidity of the anti-HA IgG raised by the three different HA DNA vaccines. Sera were analyzed from week 8 (a,b) and week 14 (c,d) in an A/PR/8/34 (H1N1)-specific NaSCN-displacement ELISA. Sera were obtained from mice inoculated. Sera were obtained from mice with sHA (open circles), tmHA (open squares) or sHA-3C3d (filled circles). Assays used pooled serum samples from each mouse group at a dilution of 1:300. Data are represented as the average of three independent experiments plus standard errors.

Figure 25

Fig. 26



[redacted] Protection from weight loss after virus challenge. At week 8 (a,b) or week 14 (c–f) mice were challenged intranasally with a lethal dose of influenza virus, A/PR/8/34 (H1N1), and monitored daily for weight loss. The data are plotted as percentage of the average initial weight. (a,c) Mice were primed and boosted with a 1 g dose of DNA vaccine. (b,d) Mice were primed and boosted with a 0.1 g dose of DNA vaccine. (e) Mice were given a single 1 g dose of DNA vaccine. (f) Mice were given a single 0.1 g dose inoculum of DNA vaccine. Sera were obtained from mice with vector (filled squares), sHA (open circles), tmHA (open squares), sHA-3C3d (filled circles), naïve-mock (open triangles) or naïve-virus (filled triangles). The open cross indicates the time-point at which all five mice in a group succumbed to disease.

Figure 26

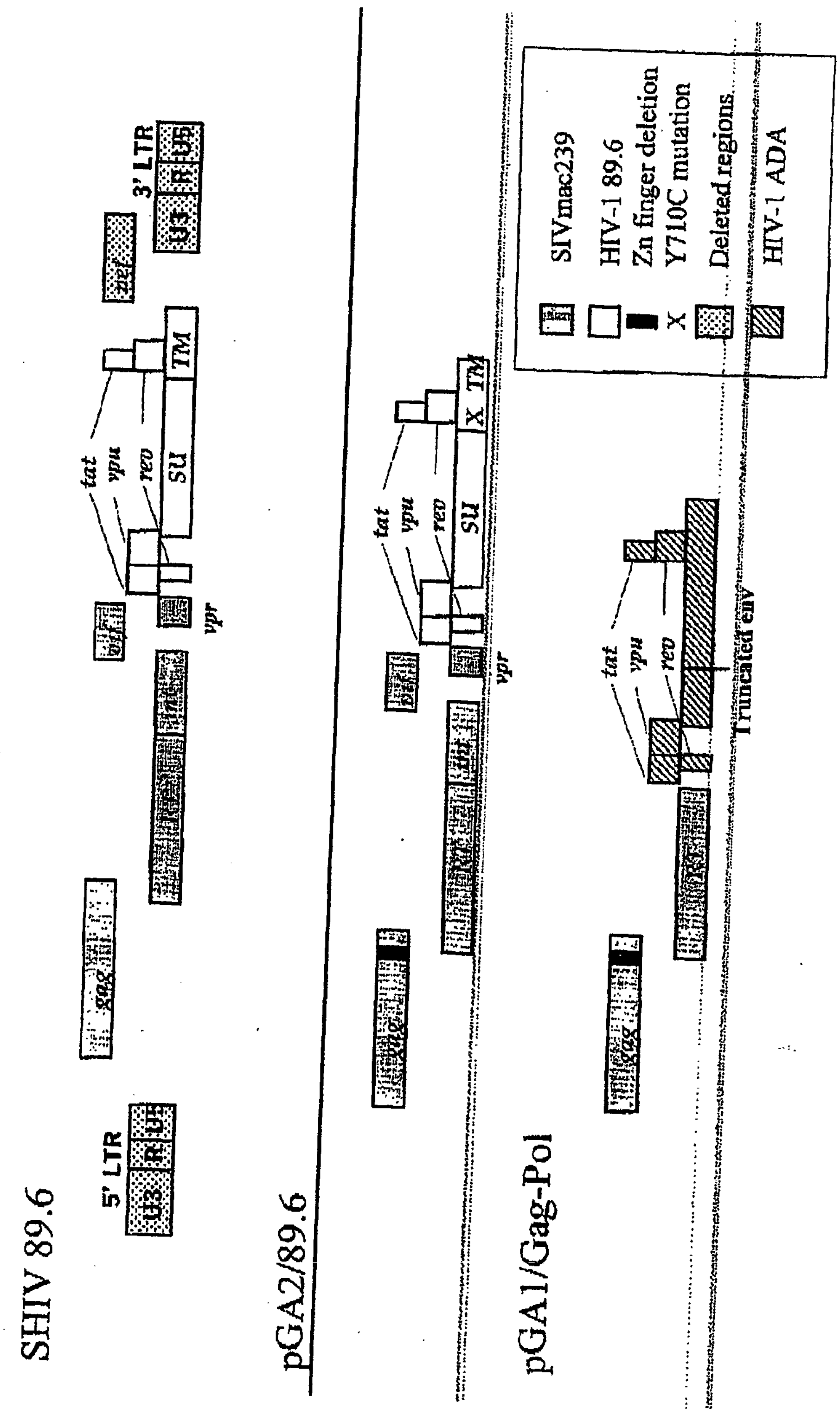

SHIV 89.6
5' LTR
U3
R
U5
gag
vif
tat
vpu
rev
vpr
SU
TM
nef
3' LTR
pGA2/89.6
X
pGA1/Gag-Pol
Truncated env
SIVmac239
HIV-1 89.6
Zn finger deletion
Y710C mutation
Deleted regions
HIV-1 ADA

FIGURE 27

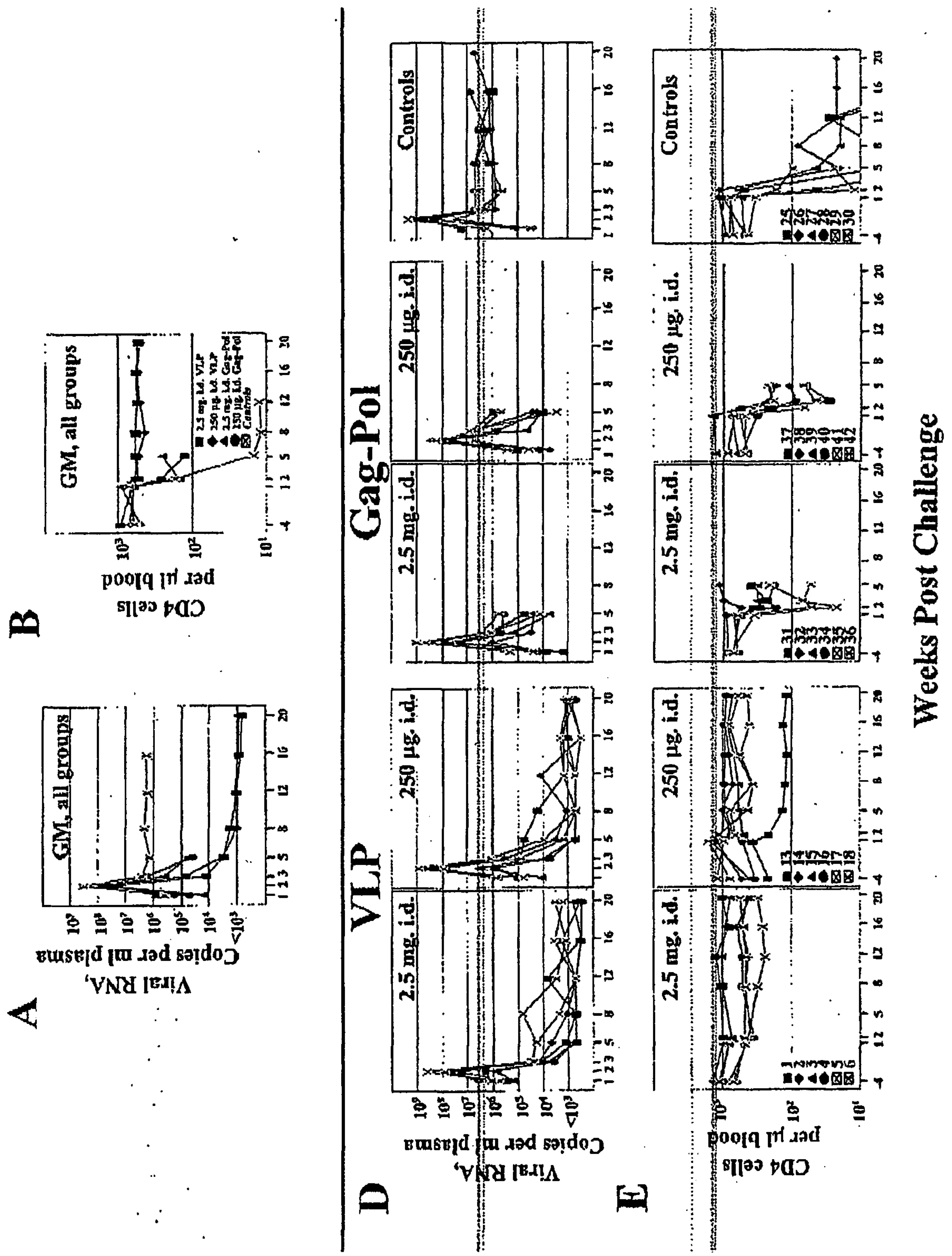

A
Viral RNA, Copies per ml plasma
GM, all groups
B
CD4 cells per µl blood
GM, all groups
VLP
Gag-Pol
D
Viral RNA, Copies per ml plasma
2.5 mg. i.d.
250 µg. i.d.
2.5 mg. i.d.
250 µg. i.d.
Controls
E
CD4 cells per µl blood
2.5 mg. i.d.
250 µg. i.d.
2.5 mg. i.d.
250 µg. i.d.
Controls
Weeks Post Challenge

FIGURE 28

Fig. 29

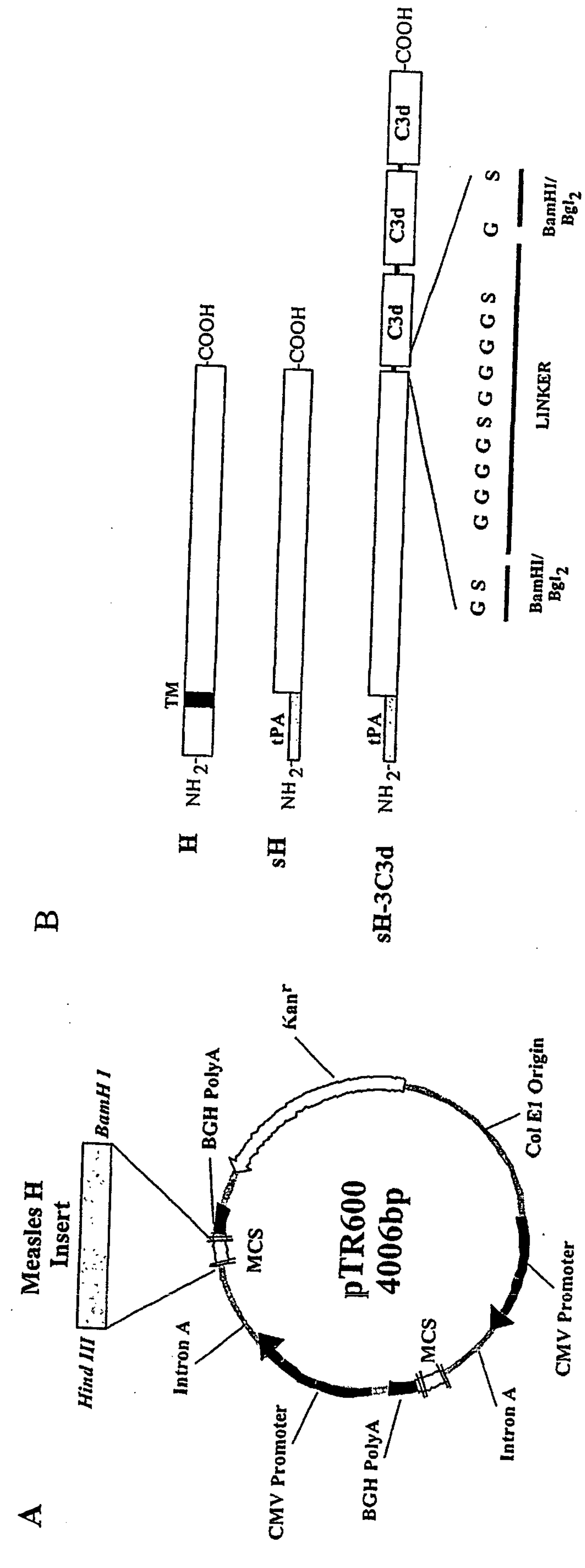

A
Measles H Insert
Hind III
BamH I
BGH PolyA
Kanr
MCS
Intron A
pTR600 4006bp
Col E1 Origin
CMV Promoter
MCS
Intron A
CMV Promoter
BGH PolyA
B
H
NH2-
TM
COOH
sH
tPA
sH-3C3d
C3d
C3d
C3d
G S
G G G G S G G G G S
G S
BamHI/ Bgl2
LINKER
BamHI/ Bgl2

Fig. 30

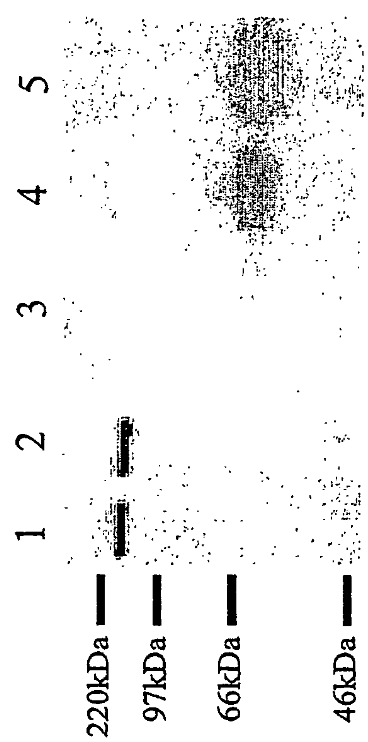
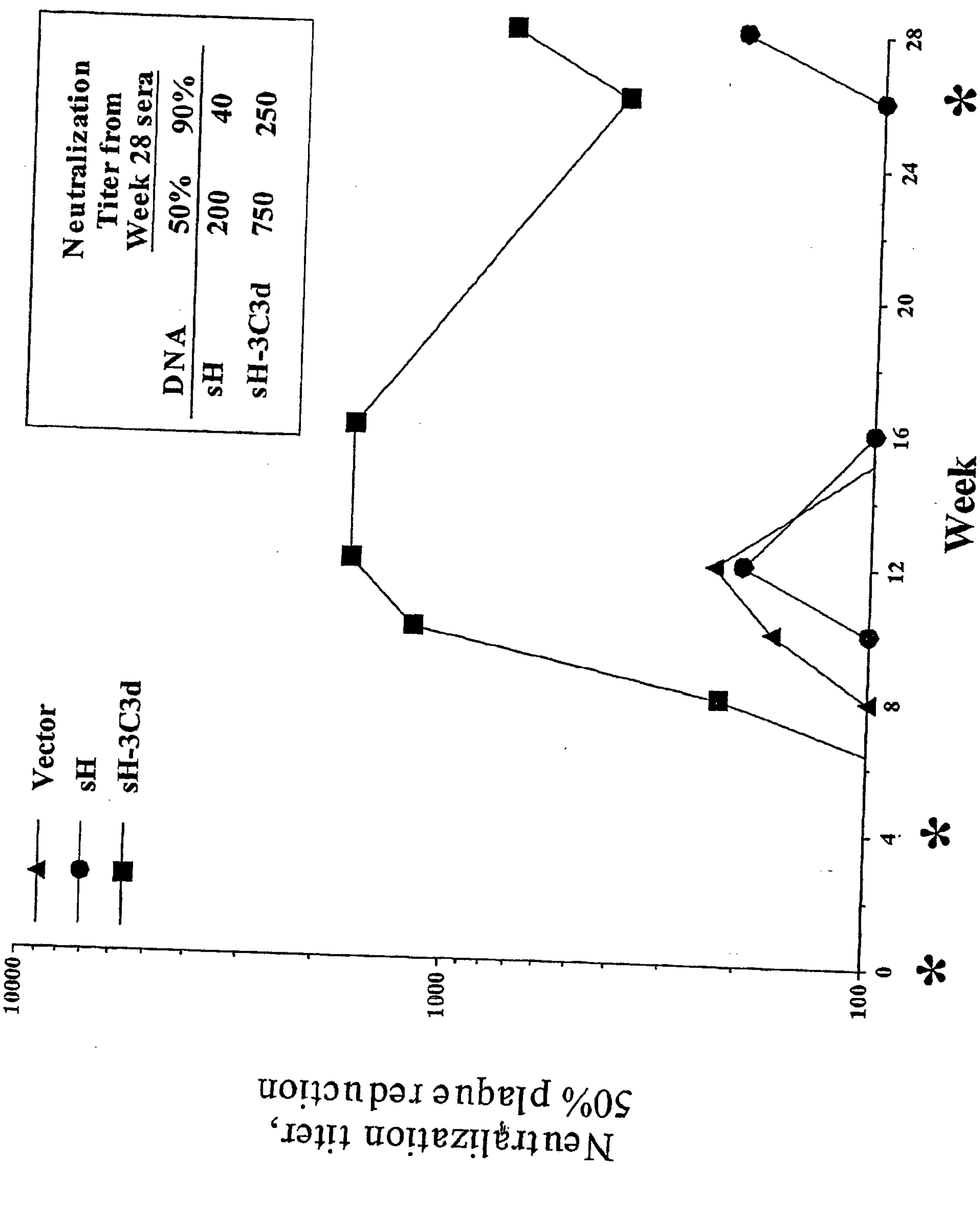


Fig. 31.


Neutralization titer,
50% plaque reduction
10000
1000
100
Week
0
4
8
12
16
20
24
28
Vector
sH
sH-3C3d
Neutralization
Titer from
Week 28 sera
DNA
50%
90%
sH
200
40
sH-3C3d
750
250
*
*
*

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PCT

(10) International Publication Number
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(22) International Filing Date: 2 March 2001 (02.03.2001)

(25) Filing Language: English

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(74) Agent: HAYZER, David, J.; Womble Carlyle Sandridge & Rice, P.O. Box 7037, Atlanta, GA 30357-0037 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report: 25 March 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: DNA EXPRESSION VECTORS AND METHODS OF USE

(57) Abstract: The present invention provides novel pGA constructs which are useful as vectors for the delivery of DNA vaccine inserts. The vaccine inserts can include the DNA transcripts of various virus, bacteria, parasite and/or fungi. Also described are methods of raising multi-epitope CD8 T-cell responses by administering therapeutically effective amounts of the novel pGA constructs comprising pathogen vaccine inserts followed by booster immunizations with a live vectored vaccine comprising the same vaccine inserts.

WO 2001/092470 A3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06795

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.2, 188.1, 207.1, 232.1; 435/455, 461, 463, 464, 466, 476, 480; 536/23.72

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, WEST 2.0

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KENT et al. Enhanced T-Cell Immunogenicity and Protective Efficacy of a Human Immunodeficiency Virus Type 1 Vaccine Regimen Consisting of Consecutive Priming with DNA and Boosting with Recombinant Fowlpox Virus. Journal of Virology. December 1998, Vol. 72, No. 12, pages 10180-10188, see entire document.	1-11, 13, 14, 16, 17, 19, 20, 22, 23 and 25-32
Y	BOHM et al. Routes of Plasmid DNA Vaccination that Prime Murine Humoral and Cellular Immune Responses. Vaccine. 1998, Vol. 16, No. 9/10, pages 945-954, see entire document.	1-11, 13-14, 16, 17, 19, 20, 22, 23 and 25-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search 28 JUNE 2001	Date of mailing of the international search report 13 JAN 2004
Name and mailing address of the ISA/US Box PCT	Authorized officer L. SCHEINER Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06795

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ROBINSON et al. Neutralizing Antibody-Independent Containment of Immunodeficiency Virus Challenges by DNA Priming and Recombinant Pox Virus Booster Immunizations. Nature Medicine. May 1999, Vol. 5, No. 5, pages 526-534, see entire document.	1-11, 13, 14, 16, 17, 19, 20, 22, 23 and 25-32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06795

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A61K 39/21, 39/275; A01N 63/00; C07H 21/04; C12N 15/74 15/86, 15/97

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/93.2, 188.1, 207.1, 232.1; 435/455, 461, 463, 464, 466, 476, 480; 536/23.72

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